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FACULTY OF PHARMACEUTICAL SCIENCES

Department of Pharmaceutical Analysis

Laboratory of Drug Quality & Registration

INSTITUTE OF INFECTION AND IMMUNITY

Department of Clinical Sciences

Academic year 2014 - 2015

High-throughput screening of antimicrobial peptides against *Candida krusei* and *Cryptococcus neoformans*

Valerie VAN LAERE

Master of Science in Drug Development

Promoter

Prof. Dr. Bart De Spiegeleer

Co-promoter

Dr. Kai Hilpert

Commissioners

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The commissioners

Name: Prof. Dr. Dieter Deforce
Address: FFW, Ottergemsesteenweg 460
9000, Ghent, Belgium

Date and signature

Name: Dr. Martijn Risseeuw
Address: FFW, Ottergemsesteenweg 460
9000, Ghent, Belgium

Date and signature

The promoter

Prof. Dr. Bart De Spiegeleer
FFW, Ottergemsesteenweg 460
9000, Ghent, Belgium

Date and signature

SUMMARY

Antimicrobial peptides are new promising alternatives in the search for new potent drugs against microorganisms due to the currently arising problem of (multi)drug resistance. They are known to have broad spectrum, whereupon they are investigated against several microorganisms, such as fungi. Fungi are the mainspring of lethal infections in immunocompromised patients. The currently existing antifungal drugs are limited, not as efficient as desired and are also subjected to resistance problems. The main objective of this research was to identify promising antimicrobial peptides active against the fungi *Candida krusei* and *Cryptococcus neoformans* by high-throughput fluorescence-based screening of two libraries of peptides.

The antifungal potential of 240 9-mer synthetic peptides and of 430 naturally occurring peptides ranging from five to seventeen amino acids in length were investigated. References to the peptides were made with their respective number in the SPOT synthesis. There were seven highly active peptides found against *C. neoformans*, of which two are from the synthetic peptide library (525 and 559) and five are from the natural peptide library (77, 221, 257, 376 and 391). Nine peptides of the natural peptide library were identified highly active against *C. krusei* (59, 77, 328, 367, 373, 375, 376, 377 and 378), while none of the synthetic peptides were. Both selective peptides against *C. krusei* (the synthetic peptide 419 and the natural peptides 77 or bactenecin-1, 367 or aurein 3.2 and 378 or uperin 3.1) or against *C. neoformans* (the synthetic peptide 559 and the natural peptide 77 or bactenecin-1) and broad spectrum peptides (the synthetic peptides 461 and 525 and the natural peptides 59 or L5K5W, 257 or temporin-HN1 and the tachyplesins or peptide numbers 375, 376 and 377) were detected.

On the other hand, the aim of the screening of the two libraries was to obtain preliminary information on important characteristics correlating to the activity profile of the peptides. A distributional and positional analysis of the amino acids within the sequences and an univariate ANOVA analysis were accomplished for this purpose. In general, it can be said that the right balance between hydrophobic and positively charged amino acids is required in order to obtain highly active peptides against the tested fungi.

SAMENVATTING

Antimicrobiële peptiden zijn beloftevolle alternatieven in de zoektocht naar nieuwe potentiële geneesmiddelen tegen micro-organismen ten gevolge van het huidige, toenemend probleem van (multi)drug resistentie. Ze zijn gekend om hun breed spectrum, waardoor ze onderzocht worden tegen verscheidene micro-organismen, zoals fungi. Fungi zijn de hoofdoorzaak van letale infecties bij immunogecomprimeerde patiënten. De huidige antifungische geneesmiddelen zijn gelimiteerd, niet zo efficiënt als gewenst en zijn ook onderworpen aan resistentie problemen. Het hoofdobjectief van dit onderzoek was het identificeren van veelbelovende antimicrobiële peptiden die actief zijn tegen de fungi *Candida krusei* en *Cryptococcus neoformans* via een op fluorescentie gebaseerde high-troughput screening van twee peptide bibliotheken.

Het antifungisch potentieel van 240 synthetische nonapeptiden en van 430 in de natuur voorkomende peptiden met een lengte van vijf tot zeventien aminozuren werd onderzocht. Zeven sterk actieve peptiden tegen *C. neoformans* werden gevonden, waarvan twee van de synthetische peptide bibliotheek afkomstig zijn (525 en 559) en vijf van de natuurlijke peptide bibliotheek (77, 221, 257, 376 and 391). Negen peptiden van de natuurlijke peptide bibliotheek waren zeer actief tegen *C. krusei* (59, 77, 328, 367, 373, 375, 376, 377 and 378), terwijl geen synthetische peptiden sterke activiteit tegen *C. krusei* vertoonden. Zowel selectieve peptiden tegen *C. krusei* (de synthetische peptide 419 en de natuurlijke peptiden 77 of bactenecin-1, 367 of aurein 3.2 en 378 of uperin 3.1) of tegen *C. neoformans* (de synthetische peptide 559 en de natuurlijke peptide 77 of bactenecin-1) als breed spectrum peptiden (de synthetische peptiden 461 en 525 en de natuurlijke peptiden 59 of L5K5W, 257 of temporin-HN1 en de tachyplesins of peptide nummers 375, 376 and 377) werden gevonden.

Daarbovenop was het doel om informatie over belangrijke karakteristieken van de peptiden te bekomen die correleren me hun activiteit. Een analyse van de distributie en de posities van de aminozuren in de sequenties en een eendimensionale ANOVA analyse werden hiervoor uitgevoerd. Algemeen kon besloten worden dat de juiste balans tussen hydrofobe en positief geladen aminozuren vereist is om sterk actieve peptiden te verkrijgen tegen de geteste fungi.

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TABLE OF CONTENTS

1. INTRODUCTION 1

1.1. ANTIMICROBIAL RESISTANCE 1

1.2. ANTIMICROBIAL PEPTIDES 2

1.3. ANTIFUNGAL PEPTIDES 3

1.3.1. Candida krusei 5

1.3.2. Cryptococcus neoformans 6

1.4. SPOT SYNTHESIS AND RESIN SYNTHESIS 6

1.5. ANTIFUNGAL SUSCEPTIBILITY TESTING 9

1.6. CELL VIABILITY FLUORESCENCE SCREENING ASSAY 10

2. OBJECTIVES 11

3. MATERIALS AND METHODS 12

3.1. GENERAL 12

3.2. SPOT SYNTHESIS OF THE PEPTIDES 12

3.3. PREPARATION OF MEDIUM AND SABOURAUD DEXTROSE AGAR PLATES13

3.3.1. Preparation of medium 13

3.3.2. Preparation of Sabouraud Dextrose Agar plates 13

3.4. PREPARATION OF THE FUNGAL STRAINS 14

3.5. PREPARATION OF THE OVERNIGHT CULTURE 14

3.6. COLONY FORMING UNITS 15

3.7. CONTROLLING THE OVERNIGHT CULTURES 16

3.8. CONTROL MINIMUM INHIBITORY CONCENTRATION ASSAY 16

3.9. CELL VIABILITY FLUORESCENCE SCREENING ASSAY 17

3.9.1. Optimization of the cell viability fluorescence screening assay............ 17
3.9.1.1. Determination of the optimal incubation time point 17
3.9.1.2. Comparison of the MIC results 18
3.9.2. Cell viability fluorescence screening assay 18
3.10. SYNTHEZING THE SELECTED PEPTIDES ON RESIN 22
3.11. MINIMAL INHIBITORY CONCENTRATION ASSAY 22
4. RESULTS 23
4.1. COLONY FORMING UNITS 23
4.2. CONTROLLING THE OVERNIGHT CULTURES 23
4.3. CONTROL MINIMUM INHIBITORY CONCENTRATION ASSAY 24
4.4. CELL VIABILITY FLUORESCENCE SCREENING ASSAY 25
4.4.1. Optimization of the screening assay 25
4.4.1.1. Determination of the optimal incubation time point 25
4.4.1.2. Comparison of the MIC results 26
4.4.2. Cell viability fluorescence screening assay 26
4.4.2.1. Activity classification of the peptides 26
4.4.2.2. Distributional and positional analysis 28
4.4.2.3. Univariate ANOVA analysis 30
4.5. MINIMAL INHIBITORY CONCENTRATION ASSAY 31
5. DISCUSSION 32
5.1. COLONY FORMING UNITS 32
5.2. CONTROLLING THE OVERNIGHT CULTURES 32
5.3. CONTROL MINIMUM INHIBITORY CONCENTRATION ASSAY 32
5.4. CELL VIABILITY FLUORESCENCE SCREENING ASSAY 33
5.4.1. Optimization of the screening assay 33
5.4.1.1. Determination of the optimal incubation time point 33
5.4.1.2. Comparison of the MIC results 34

5.4.2. Cell viability fluorescence screening assay 34
5.4.2.1. Activity classification of the peptides... 34
5.4.2.2. Best peptides and their broad spectrum activity 35
5.4.2.3. Distributional and positional analysis 39
5.4.2.4. Univariate ANOVA analysis 42
5.4.3. Limitations 44
5.5. MINIMAL INHIBITORY CONCENTRATION ASSAY 45
5.6. TIME LIMITATION AND FUTURE WORK.. 46
6. CONCLUSION 47
7. ACKNOWLEDGEMENT.. 49

ABBREVIATIONS

AFP(s)	antifungal peptide(s)
AMP(s)	antimicrobial peptide(s)
AFST	Antifungal Susceptibility Testing
CFU	Colony Forming Units
C. albicans	*Candida albicans*
C. krusei	*Candida krusei*
C. neoformans	*Cryptococcus neoformans*
DMSO	dimethylsulfoxide
EUCAST	European Committee on Antimicrobial Susceptibility Testing
Fmoc	9-fluorenylmethyloxycarbonyl chloride
HIV	Human Immunodeficiency Virus
HPLC	high-performance liquid chromatography
IC75	75% inhibitory concentration
IUB	International Union of Biochemistry
IUPAC	International Union of Pure and Applied Chemistry
(m)M	(milli)molair
MIC(s)	minimal inhibitory concentration(s)
MOPS	3-(N-morpholino)-propanesulphonic acid
MRSA	methicillin-resistant *Straphylococcus aureus*

NADPH + H^+	reduced nicotinamide adenine dinucleotide phosphate
nm	nanometer
NMR	Nuclear Magnetic Resonance
OD	optical density
P. auruginosa	*Pseudomonas aeruginosa*
rfu	relative fluorescence units
spp.	species
SPPS	solid phase peptide synthesis
t-Boc	di-tert-butyl dicarbonate
var.	variety
VRE	vancomycin-resistant *Enterococcus*
WHO	World Health Organization

1. INTRODUCTION

1.1. ANTIMICROBIAL RESISTANCE

One of the biggest, currently arising medicinal problems regarding treatment of infections caused by microorganisms is (multi)drug resistance. The World Health Organization (WHO) depicts this as an expanding risk at a global level.[1] Antimicrobial resistance implies antimicrobial drugs which are no longer capable of effectively killing or inhibiting the growth of various microorganisms. The microorganisms can evolve and mutate their genes by many mechanisms in a way that they can circumvent the effect of the drug.[1] The use, and especially mis- and overuse of the current antimicrobial drugs increases the rate of resistance and the worldwide threat of it. Antimicrobial resistance includes resistance in bacteria, parasites, viruses and fungi.[1,2]

Especially antibiotic resistance, occurring in bacteria, is escalating on a large scale. This makes treatment more challenging, above all for the hospital-acquired infections and mainly the ones caused by multidrug resistant bacteria. Well-known examples are methicillin-resistant *Straphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE).[1,2] The same problem is happening to antifungal drugs. Mainly invasive fungi, like *Candida* or *Aspergillus*, are becoming resistant to several antifungal agents.[3]

Because of the rising problems resulting from the failure of treatment of infections caused by these microorganisms, there is an increasing interest in investigating new possible antimicrobial drugs, vaccines and alternative therapies.[2] The most significant requirements of the new alternatives are a broad activity spectrum or a specific activity against for example multidrug resistant microorganisms and a low toxicity against the host cells. One of the promising alternatives are the antimicrobial peptides (AMPs).[2,4]

1.2. ANTIMICROBIAL PEPTIDES

AMPs are a large group of peptides naturally occurring in various animal, plant and microorganism species.[4] Some of them participate in the innate immune system.[5] They are generated by various tissues and cell types. AMPs are normally found in locations which are frequently exposed to microbes, for example the mucosae. Their activity and production is induced by inflammation or trauma.[5,6] In general, they contain approximately 10 to 50 amino acids.[4,5] They usually have dissimilar sequences but share often similar biophysical and biochemical properties, such as a rather hydrophobic and amphipathic structure. Because of properties like amphipathicity, positive charge, size and amino acid composition, most peptides can interact with bacterial membranes and some form stable or transient pores in microbial membranes.[4,5,6] Not only has the membrane-active functionality of the peptides contributed to their activity profile but other aspects as well, such as the inhibition of the protein synthesis and the inhibition of the enzymatic activity.[5]

In the beginning of 1960, multidrug-resistant microbes became an increasing problem and the efficiency of antibiotics was decreasing. This was the starting point for further research in AMPs. In 1962, the first animal AMP, bombinin, was isolated from the frog *Bombina variegate*.[7,8] Later on, the discovery of lactoferrin from milk, α-defenisins from leukocytes of rabbits and humans and some other small AMPs took place.[7,8]

A breakthrough came in 1981, with the isolation of the AMPs P9A and P9B out of the hemolymph of the pupae of a silk moth, *Hyalophora cecropia*, after injection of bacteria into the pupae by Boman *et al*. They are described as the first accounted α-helical AMPs and were later renamed cecropins.[7] From then on, a lot of other research led to other important discoveries, for example in 1987 with the isolation and characterization of magainins from the frog *Xenopus laevis*,[7] some years later of the β- and θ-defensins[7] and around 1995 of the first anionic AMPs.[8] At the same time, it was clarified that AMPs are mainly significant in organisms with a suppressed adaptive immune system. However, they are present in almost every eukaryotic species.[7,8]

AMPs are now studied as possible replacement for the classical antimicrobial drugs against infections caused by antibiotic-resistant microorganisms. They have several advantages, including their broad spectrum, rapid activity and the low occurrence of resistance.[8] They can also work synergistically with common antibiotics. Better perception of the activity in model membranes has resulted in optimization of the AMPs.[8,9]

1.3. ANTIFUNGAL PEPTIDES

Antifungal peptides (AFPs) are a subclass of antimicrobial peptides, which are specified against fungal infections. Their relevance is rising due to higher risk infections with opportunistic fungi like *Aspergillus fumigatus, Cryptococcus neoformans* and *Candida albicans*.[11,12] This is the result of different aspects, but mostly because of the increased number of immunocompromised individuals. The main target for these invasive fungal infections are patients with a suppressed immune system, such as patients affected by cancer or an organ transplantation, as well as Human Immunodeficiency Virus (HIV) patients.[13,14] Antifungal resistance is also becoming a substantial problem, especially in patients under long-lasting or prophylactic treatment. Some fungal strains are naturally resistant to several antifungal agents, others develop a lower susceptibility because of incorrect use of them.[13,14]

The currently existing antifungal agents are limited and not as efficient as desired. Some of them have targets which are present in human cells and can therefore be toxic.[13] They can be classified based on their working mechanisms, which are: the polyene macrolides bind with (ergo)sterols in the fungal cell membrane which results in loss of cell content, the azole derivates inhibit 14-α-lanosterol demethylase which is a crucial enzyme in the conversion to ergosterol, the allylamines inhibit squalene epoxidase which is also necessary for the ergosterol biosynthesis, the echinocandins inhibit 1,3-β-glucan synthase and therefore the biosynthesis of glucan and others such as griseofulvin, benzoic acid and ciclopirox.[6,13]

There is a large interest in finding alternatives for fungal infections. AMPs are already proven to have activity against gram positive and gram negative bacteria, fungi, parasites and enveloped viruses.[12] Because of their broad activity, there is a good chance that some of these peptides can be used as against fungal infections. With some of them in clinical trials and others still being preclinically tested, there is a broad interest in further research and optimization of yet undiscovered AMPs or novel AMPs.[11,12]

The mode of action of the AFPs is not completely declared yet.[12,15] The general accepted theory for the mode of action of AMPs is that it is directly related to the structural elements defining the cell surface. Positive residues in the peptide sequence are more likely to electrostatically interact with the negatively charged lipids appearing in the cell membranes of bacteria and fungi, contrary to the more neutral phospholipids on the exterior of mammalian cells.[16] This gives the AMPs the desired toxic selectivity to the pathogens. Not only the cationic nature but also the secondary structure is found to be crucial in binding to the negatively charged components.[16]

Since fungi are eukaryotic, they differ from the prokaryotic bacteria in cell structure. For example, fungi have besides the cell membrane also a cell wall. The cell wall consists of a chitin layer, a β-glucan network and a most exterior layer covered with proteins. These proteins can form disulfide bridges and are normally glycolysated.[17] These features add up in making the cell wall a strong barrier. The plasma membrane from bacteria and fungi both include anionic lipids but phosphatidylglycerol and cardiolipin are more common in the bacterial cell membrane, while phosphatidylserine and phosphatidylinositol are more present in fungi.[17] Sterols are also incorporated in eukaryotic plasma membranes, with cholesterol for animal membranes and ergosterol for lower eukaryotes such as the fungi. The focus for AMPs was mainly set on bacteria, which is why the antibacterial mode of action is understood better. This is in contrast to the antifungal activity of these peptides, which is not completely elucidated yet. The peptides can bind to the fungal cell wall, damage the cell membrane and thereby kill the fungal cell.[17]

Primarily amphipatic peptides, this means including both a positive charge and a neutral hydrophobic part in the sequence, are capable to insert in the plasma membrane. It is also possible that the peptide is internalized, for example by an efflux pump or receptor-mediated, where it interacts with intracellular targets or with a signaling cascade.[17]

Some of the AMPs have shown to have an immunomodulatory effect, besides their antimicrobial activity.[15] Examples are accelerated wound healing, attraction of different immune cels and regulation of cytokines and chemokines production. This can be very interesting for fungal diseases, since they primarily appear in people with a compromised immune system.[15]

1.3.1. *Candida krusei*

Candida krusei (*C. krusei*) is a rare pathogen, which can primarily invade mucosae. It is a gram positive yeast.[18] In the last decades, its relevance has been rising due to diseases like fungaemia, endophtalmitis, arthritis and endocarditis caused by this organism. *C. krusei* infections generally appear in hospitals and in immunocompromised patients.[19] It is identified as a possibly multidrug resistant organism since it is naturally resistant to fluconazole and weakly susceptible to amphotericin B and flucytosine.[19] Secondary infections with *C. krusei* have been reported in patients treated by previous mentioned drugs.[19]

Candida krusei is slightly different in its structure, metabolism and defense mechanisms from other *Candida* species (spp.). For example, it has a longer form instead of the normal *Candida* egg-shaped form,[20] the genes are located on eight chromosomes comparing to sixteen for *Candida albicans* and its reproduction is more tending to a sexual one instead of the normal asexual *Candida* spp.[18] All these features can add up to the different behavior of *C. krusei* and to the discussion about its reclassification.[18] *C. krusei* exists in two conditions, as yeast and as pseudohyphae. Their separation can be tricky since they normally grow together in cultures. *C. krusei* is a glucose fermenter and can easily be distinguished from other *Candida* yeasts because it does not grow as spheres on Sabouraud Dextrose medium but as spreading colonies.[20]

1.3.2. *Cryptococcus neoformans*

Cryptococcus neoformans (*C. neoformans*) is an encapsulated pathogenic gram positive yeast from the genus *Cryptococcus* and is especially harmful in immunocompromised patients.[21] Transplant recipients and HIV-positive patients are highly susceptible to a *C. neoformans* infection, resulting in diseases such as cryptococcosis.[22] This microorganism is highly abundant and therefore many people are exposed to it. The highest rate of mortality caused by this organism takes mainly place in the sub-Saharan part of Africa.[23] The transmission essentially takes place via the respiratory tract.[24] There are different recommendations in treatment depending on the underlying cause and the anatomic appearance of the patient.[22] Resistance in clinical *C. neoformans* samples against antifungal agents is a rather rare phenomena but has been recently evolved to a larger concern, especially in patients who need prolonged treatment.[23]

C. neoformans can reproduce in both an asexual and a sexual way.[24] Its virulence is mainly originated from its polysaccharide capsule.[22] It is known to have a slow rate of mutation.[25] *C. neoformans* forms white-beige colonies on Sabouraud Dextrose Agar. They have a mucous appearance resulting from the capsule enveloping the fungal cells.[22] It is a non-fermentative yeast.[21] Two varieties (var.) are found, var. *neoformans* and var. *gatti*. They differ in their genetics, ecology, life cycles and physiology.[21]

1.4. SPOT SYNTHESIS AND RESIN SYNTHESIS

SPOT synthesis is a variation on Solid Phase Peptide synthesis (SPPS). In SPPS, the amino acids are stepwisely coupled to reactive functional groups of a polymer, such as resin.[26] The principle of SPPS is illustrated in figure 1.1. The polymer with attached functional groups will react with the free carboxyl group of the first amino group-protected amino acid. The amino termini protecting group is removed and a new free reactive amino group is generated, which can now covalently bind with the second amino group-protected amino acid. After a few cycles, the desired peptide is formed.[26,27,28]

The most commonly used protecting groups for the α-amino group of the amino acid in SPPS are 9-fluorenylmethyloxycarbonyl chloride (Fmoc) and di-tert-butyl dicarbonate (t-Boc). Fmoc will be used for the synthesis of the peptides in this research project because it has several advantages compared to the other protection group. One of them is that it requires milder conditions during the cleavage.[29]

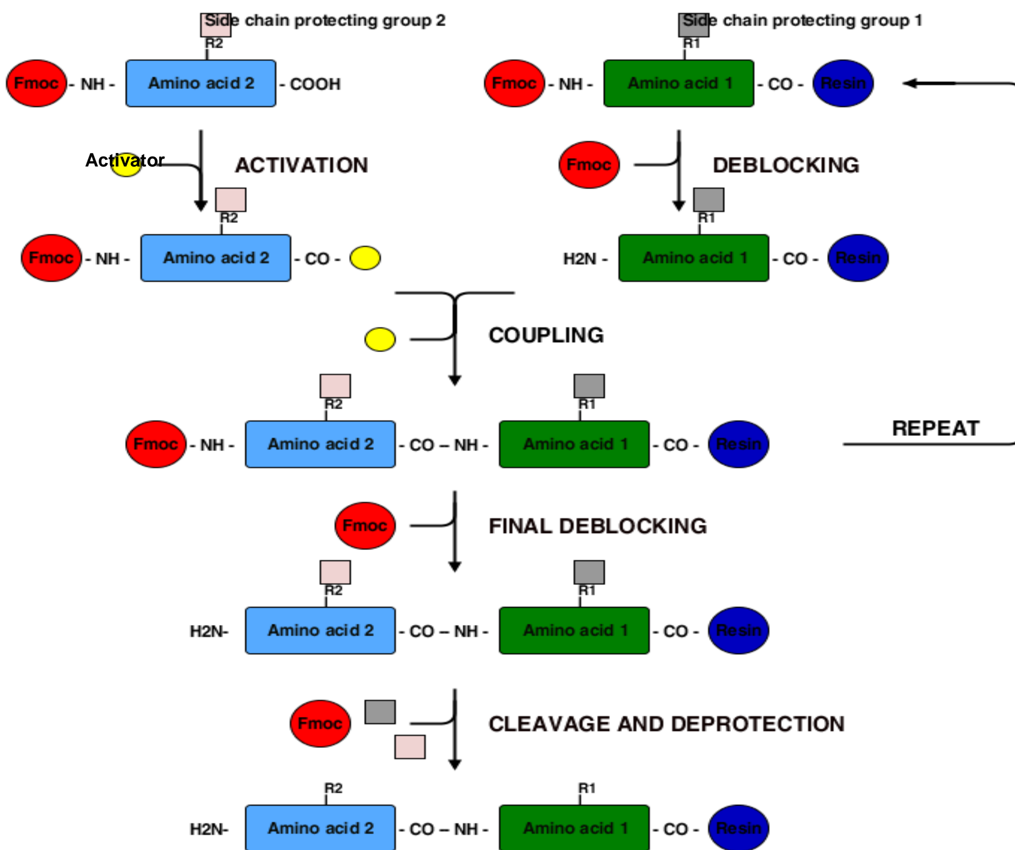


Figure 1.1.: Schematic representation of the peptide synthesis using Fmoc-based chemistry. The amino acids are protected at their amino termini with Fmoc groups and at some of their side chain groups to avert side reactions. The amino acids are then activated to prepare them for the peptide bonding. The amino acids derivatives are coupled on resin beads and peptide bonds are formed. These steps can be repeated until the desired peptide sequence is formed. Finally, the Fmoc group is removed and a side chain deprotection step and cleavage from the resin beads is performed.[26,27,28]

The advantages coming with SPPS are numerous, including the simplicity and the fact that the whole process can be fully automated.[30] But because this method was still not fast enough and rather expensive, researchers kept pursuing new and better techniques for peptide synthesis. One of these techniques is the SPOT synthesis.[30,31]

The SPOT synthesis technique was developed by Ronald Frank and his colleagues in 1990.[32] Further automation was developed by the Jens Schneider-Mergener group.[30] It is usually performed on cellulose membranes, due to its low price and safety and stability in acidic, organic and aqueous solvents in comparison to other materials. It can be performed manually or (semi-)automatically by using pipetting robots. There are three important steps in SPOT synthesis: the treatment of the membrane surface, linking of the amino acids and removing the protecting groups.[4,30,31]

SPOT synthesis is a commonly used technique to synthesize and analyze a large number of peptides on absorptive membranes. Small droplets of preactivated amino acid derivatives are dispensed on the porous membrane, which absorbs these, followed by the chemical coupling reaction.[30] The advantages of this method consist of being fast, accessible in use and economical in comparison to most standard SPPS methods on resin; which are generally more expensive and can synthesize only a limited number of peptides.[31] SPOT synthesis can give preliminary information on the activity of hundreds to thousands of sequences. Therefore, it is widely used for the preparation of large libraries of peptides from which peptide-based drug candidates can be identified.[30]

Two peptide libraries synthesized using the SPOT synthesis technique are investigated for this thesis. The first one is containing 240 synthetic peptides. These are already been optimized to be antimicrobial and non-hemolytic after testing them against *Pseudomonas aeruginosa* (*P. aeruginosa*). They have a length of nine amino acids and will be further referred as the synthetic peptides. The next one is a library containing 430 short peptides, derived from a database "The Antimicrobial Database 2" containing naturally occurring antimicrobial peptides.[10] They consist of five to seventeen amino acids and will be further referred as the natural peptides.

The peptides are only synthesized in small amounts and purification is not possible.[4] However, the low yield and purity is not affecting the results heavily and the peptides can still be used for a first screening. In previous studies, assessments of the quantity of contaminants were identified less than 30%.[4] In order to receive more precise information for the sequences of interest selected after the first screening, SPPS on resin should be performed.[32] It has the disadvantage to be more expensive and time and work consuming, compared to the SPOT synthesis method but a higher yield and purity is obtained and the exact concentration of the peptides can be determined.[4]

1.5. ANTIFUNGAL SUSCEPTIBILITY TESTING

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) is constructed by the European Society of Clinical Microbiology and Infectious Diseases and European national breakpoint committees.[33] EUCAST strives to the standardization of antimicrobial breakpoint and susceptibility testing techniques. EUCAST constituted a Subcommittee on Antifungal Susceptibility Testing (AFST) that focuses on antifungal agents.[33] A broth dilution method is proposed for determining the minimal inhibitory concentration (MIC) of fungi.[33] The MIC is the lowest concentration in µg/mL of an antimicrobial drug needed to inhibit the visible growth of the organism under specific circumstances.[33] The MIC values are used both for susceptibility testing of microorganisms to different antimicrobial agents and for estimating the activity of new potential drugs.[33,34]

Standardization of the fungal colony forming units (CFU) in a certain volume is crucial for the accuracy and reproducibility of the results of the different tests executed. The CFU is the amount of living and growing microorganisms in a solution, assuming that each organism can form a colony once transferred onto solid media.[33] An inoculum is the CFU in a defined volume and is expressed as CFU/mL. EUCAST aims for a final inoculum between $0,5 \times 10^5$ and $2,5 \times 10^5$ CFU/mL for antifungal susceptibility testing.[33] This is verified by measuring the optical density (OD) of fungal overnight cultures prior the start of an experiment.

The OD is correlated with the inoculum and is therefore used as a quick and rough control of this concentration in order to decide which dilution of the overnight culture is needed to achieve the desired final inoculum.[33,34] This is confirmed by performing a plate count on a control plate after the experiment is finished.[33]

1.6. CELL VIABILITY FLUORESCENCE SCREENING ASSAY

The peptides are screened by a cell viability assay using the dye resazurin. Resazurin or 7-hydroxy-3H-phenoxazin-3-one-10-oxide is a blue dye used as an indicator to estimate the amount of viable cells. It has a low intrinsic fluorescence. Viable fungal cells can reduce it to the pink, fluorescent resorufin.[35] An illustration of this reduction can be found in figure 1.2. This is probably executed by mitochondrial enzymes, who transfer the electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH + H^+) to resazurin.[35] If the cells are killed by the peptides, they are no longer capable of executing this reduction and can no longer generate a fluorescent signal. This reduction can be measured as a change of fluorescence since the absorbance maximum changes from a wavelength of 605 nanometer to 573 nanometer (nm). The number of viable cells correlates to the fluorescence emitted.[35,36]

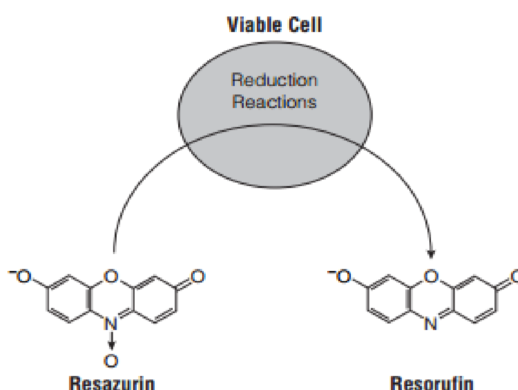


Figure 1.2.: Reaction scheme of the reduction of the dye resazurin to resorufin by viable cells used in the cell viability fluorescence screening assay. This reduction can be measured as a change in fluorescence, correlating with the amount of viable cells.[37]

2. OBJECTIVES

The main objective of this research is to identify promising antimicrobial peptides active against *Candida krusei* and *Cryptococcus neoformans* by high-throughput screening of two peptide libraries. Firstly, the focus is laid on investigating the antifungal potential of 240 9-mer synthetic peptides. This synthetic library was initially optimized for its activity against *Pseudomonas aeruginosa* and its low toxicity against red blood cells. Based on the fact that antimicrobial peptides possess broad-spectrum activity, the action of those synthetic peptides is explored against the fungal strains mentioned above. Additionally, the potential of 430 naturally occurring peptides ranging from five to seventeen amino acids in length is studied.

On the other hand, the aim of the screening of the two libraries is not only to identify strong acting antifungal peptides, but also to obtain preliminary information on important characteristics correlating to the activity profile of the peptides. Examples of these features are the distribution and the position of different amino acids in the peptide sequences. These characteristics can then be used in the design of optimized peptides with improved properties.

Different steps are conducted to achieve the main objective:

- Examining the antifungal activity of fluconazole and peptide X (AMP with proven antifungal activity) against *Candida krusei* and *Cryptococcus neoformans.*
- Optimization of the screening assay towards *Candida krusei* and *Cryptococcus neoformans.*
- Screening of the synthetic and the natural peptides, synthesized by the SPOT synthesis method, against *Candida krusei* and *Cryptococcus neoformans.*
- Resin synthesis and minimal inhibitory concentration (MIC) determination of selected peptides from the screening assay against *Candida krusei* and *Cryptococcus neoformans*.

3. MATERIALS AND METHODS

3.1. GENERAL

All the experiments were executed under safety protocol. Lab coats and gloves were used while working in the laboratories. The experiments were carried out under a class II microbiological safety cabinet (Walker, Glossop, U.K.). The working areas and apparatus used were sterilized using 70% ethanol to reduce risk of contamination. Media and most of the materials were autoclaved prior to use. Experimental wastes were disposed in the correct waste bins, which were then autoclaved. The *Candida krusei* and *Cryptococcus neoformans* strains were autoclaved after use and then thrown away in a clinical waste container. More detailed information about the traceability of the materials can be found in the lab notebook of Valerie Van Laere.

3.2. SPOT SYNTHESIS OF THE PEPTIDES

Peptide synthesis is not included in the thesis due to the limited amount of time available. The peptides are kindly provided by the group of Dr. Kai Hilpert. The machine used for the SPOT synthesis is the MultiPep RSI (Intavis Bioanalytical Instruments, Cologne, Germany) which is an automated multiple synthesizer containing a pipetting robot runned by a computer software programme. The protocol of the MultiPep RSi Manual from the manufacturer Intavis Bioanalytical Instruments is followed. References to the peptides in this thesis are made with their respective number in the SPOT synthesis. The sequences of the most interesting peptides can be found in table 5.1.

3.3. PREPARATION OF MEDIUM AND SABOURAUD DEXTROSE AGAR PLATES

3.3.1. Preparation of medium

The medium used for both fungi is an RPMI-1640 containing medium. It is a synthetic medium which contains the buffer 3-(N-morpholino)-propanesulphonic acid (MOPS) with a concentration of 0,185 mol/L and glucose with a concentration of 20 g/L. This is a relatively high glucose concentration for culturing fungi but a better fungal growth was proven using a 20 g/L concentration, compared to the normal 2 g/L glucose content in RPMI-1640.[30] A standard protocol conforming to the EUCAST criteria is followed.[30] 10,40 g of RPMI-1640 (Sigma-Aldrich, St. Louis, U.S.A.), 34,53 g of MOPS (Sigma-Aldrich, St. Louis, U.S.A.) and 18 g of D-(+)-glucose monohydrate (Merck, Darmstadt, Germany) are added to a beaker of 1 L. The beaker is filled until approximately 900 mL with demineralized water and placed on the magnetic stirrer (Rigal-Bennett, East Yorkshire, U.K.). The pH of the content of the beaker is verified with a pH meter (Mettler-Toledo, Barcelona, Spain) and corrected to a pH of ± 7,0 at 25°C by adding 10 M sodium hydroxide (Sigma-Aldrich, St. Louis, U.S.A.). Then, the beaker is filled up to 1 L. Afterwards, the medium is filtered through a Nalgene Syringe 0,2 µm filter (ThermoFisher Scientific, Waltham, U.S.A) with a Luer-Lok syringe (BD Plastipak, New Jersey, U.S.A.) in laboratory bottles. These are stored in the cold room at 4°C. This medium will be further referred as RPMI medium.

3.3.2. Preparation of Sabouraud Dextrose Agar plates

A 65 g/L demineralized water solution of Sabouraud Dextrose Agar (ThermoFisher Scientific, Waltham, U.S.A) is prepared as recommended by the manufacturer ThermoScientific. The solution is autoclaved (Boxer Laboratory Equipment Ltd, Ware, U.K.) for 20 minutes at 121°C. After cooling down, the solution is poured in sterile empty petri dishes (ThermoFisher Scientific, Waltham, U.S.A). The Sabouraud Dextrose Agar plates are stored in the cold room at 4°C.

3.4. PREPARATION OF THE FUNGAL STRAINS

The *Candida krusei* and *Cryptococcus neoformans* strains are obtained from clinical samples from St. George's NHS Trust. Two Sabouraud Dextrose Agar plates are streaked with both samples and the plates are incubated in the Air-Jacketed DH Autoflow Automatic incubator with 0% CO_2 (NuAire, Plymouth, U.S.A) at 30°C for 24 hours. Two overnight cultures are made by taking four to five colonies from the plates and dissolving them in 3 to 4 mL RMPI medium in glass test tubes. After approximately 24 hours in the incubator shaker Incu-Shake Mini (SciQuip, Shropshire, U.K.) at 30°C, 1 mL of these fungal cultures and 70 µL dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, U.S.A.) are transferred into cryogenic vials using pipettes (Mettler-Toledo, Barcelona, Spain) and are stored in the freezer (Eppendorf, Hamburg, Germany) at -80°C. They are from now on used as cryostocks for the rest of the work carried out for this thesis. DMSO is a cryoprotectant, this is a compound which protects cells against freezing impairment. It works by preventing the forming of disruptive ice crystals because it lowers the freezing point of the liquid mixture.[38]

3.5. PREPARATION OF THE OVERNIGHT CULTURE

The two fungi are inoculated from the cryogenic vials on two separate Sabouraud Dextrose Agar plates. After 24 hours in the incubator at 30°C, the overnight cultures are prepared. Four to five colonies of each plate are taken with a loop and dissolved in 3 to 4 mL RPMI medium in two separate glass test tubes. They are incubated overnight in the incubator shaker at 30°C. The overnight cultures have to be remade every day, the streak plates every week.

3.6. COLONY FORMING UNITS

To determine the CFU, a dilution series is made starting from the overnight cultures. Firstly, 150 µL of the overnight culture is added in an Eppendorf tube (Eppendorf AG, Hamburg, Germany). Then, 900 µL of RPMI medium is added in seven other Eppendorf tubes. 100 µL of the first Eppendorf tube is taken and added in the next Eppendorf tube. This results in a 10^{-1} dilution. After mixing the Eppendorf tube in the vortex Varimix (SciQuip, Shropshire, UK), 100 µL of the second Eppendorf tube is taken and added to the third one, which is mixed. This process is repeated for all the Eppendorf tubes. This results in a series of one in ten dilutions. This is done for both fungi. After the dilution series is completed, five drops of 5 µL of each dilution step are spotted on the predesignated section of a Sabouraud Dextrose Agar plate. An illustration of this can be found in figure 3.1. Then, the plates are incubated for 24 hours at 30°C.

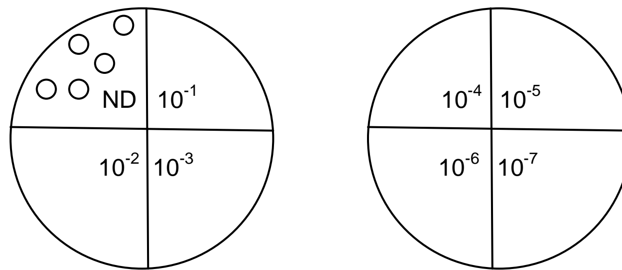


Figure 3.1.: Illustration of Sabouraud Dextrose Agar plates used for determining the CFU/mL of *C. krusei and C. neoformans*. Every section contains five drops of 5 µL of one of the ten-fold serial dilutions, in particular a non-diluted (ND) section and the dilutions from 10^{-1} to 10^{-7}.

The colonies are counted the next day in the countable dilution section and the CFU/mL is calculated with the following formula 3.1:

$$\text{CFU/mL} = \frac{(\text{Sum of the colonies in the chosen } 10^{-x} \text{ dilution section / 5) CFU}}{\frac{0{,}005 \text{ mL}}{10^{-x}}} \quad (3.1)$$

3.7. CONTROLLING THE OVERNIGHT CULTURES

After overnight incubation at 30°C, the OD of the overnight cultures is measured with an Ultraspec 2100 Pro UV/VIS spectrophotometer (Amersham Biosciences, Diegem, Belgium) at a wavelength of 600 nm. 1 mL RPMI medium is pipetted in a cuvette, which is used as blank. 900 µL RPMI and 100 µL of both overnight cultures are added in two separate cuvettes. The spectrophotometer is set on the absorbance program with a wavelength of 600 nm, after which the OD is measured.

A control plate is made after the assay to double-check if the fungal concentration is still in the desired range. A ten-fold serial dilution of the diluted overnight culture, of which is assumed that the concentration is approximately 0,5-2,5 x 10^5 CFU/mL, is prepared,. Five drops of 5 µL of each dilution step are spotted on a Sabouraud Dextrose Agar plate, which is then incubated overnight at 30°C. The colonies are counted the next day in the countable dilution section and the final inoculum is calculated with formula 3.1.

3.8. CONTROL MINIMUM INHIBITORY CONCENTRATION ASSAY

The following two drugs have been chosen for this control experiment: peptide X, which is a peptide from the stock peptides in the lab of the group of Dr. Kai Hilpert that has been previously found to be active against *C. krusei* and *C. neoformans*, and fluconazole, which is applied as standard therapy against many fungal infections. Peptide X is included as a validation for the cell viability screening assay, in which the MIC is determined using a fluorescence reader instead of visual determination.

A solution with a concentration of 250 µg/mL for fluconazole and 100 µg/mL for peptide X is prepared. A two-fold dilution series on a transparent 96-well microtitre plate (Corning Incorporated, New York, U.S.A.) is completed for both drugs in the 0,5-2,5 x 10^5 CFU/mL *C. krusei* and *C. neoformans* solutions using a electronic multichannel pipette (Mettler-Toledo, Barcelona, Spain). A positive control, which is the 0,5-2,5 x 10^5 CFU/mL diluted overnight culture, and a negative control, which is the RPMI medium, are also included on the 96-well microtitre plate. The 96-well plate is then incubated for 24 hours at 30°C. Three biological repeats of the assay are performed.

3.9. CELL VIABILITY FLUORESCENCE SCREENING ASSAY

3.9.1. Optimization of the cell viability fluorescence screening assay

3.9.1.1. Determination of the optimal incubation time point

Firstly, the resazurin-based fluorescence screening assay is optimized using peptide X in order to find the optimal conditions for both fungi and the peptides. In attempt to optimize this assay, a time-course experiment with the dye resazurin and the fungal cultures as a positive controls is conducted. By using the fluorescence detection expressed in relative fluorescence units (rfu), the optimal time point is determined that would show a clear difference between the wells where growth is not observed and the wells where the culture is growing. The results are read with the fluorescence microplate reader at different time points, in particular 0, 2, 4, 6 and 17 hours.

The settings for the Infinite F200 PRO multifunctional microplate reader (Tecan, Männedorf, Switzerland) are 60 seconds shaking in an orbital with an amplitude of 4 mm with one minute pause. The temperature is 30°C. The fluorescence settings are an excitation wavelength of 485 nm, an emission wavelength of 535 nm, a gain of 40, an integration time of 20 µs and a lag time of 0 µs.

100 µL of the diluted overnight culture with a final inoculum of 0,5-2,5 x 10^5 CFU/mL are added to the first 10 columns in one row for each fungus in a black 96-well microtitre plate (Greiner bio-one, Frickenhausen, Germany). An extra 50 µL is added in the first well in the first column. 50 µL of a 2000 µg/mL solution of peptide X is then also added to the first well. A serial dilution is then performed from column 1 to 10. Column 11 is the negative control and is loaded with the growth media only. Column 12 is the positive control and is loaded with the diluted overnight cultures. Lastly, 10 µL of a 500 mM resazurin solution (Sigma Aldrich, St. Louis, U.S.A.) is added in every filled well in the 96-well microtitre plate. The rfu is measured by a fluorescent microplate reader at the different time points. Between these time points, the 96-well microtitre plates covered with an adhesive sealing film are incubated at 30°C. The rfu measurements are recalculated to percentage fluorescence using formula 3.2.

$$F_{percentage} = \frac{F_w - F_{0\%}}{F_{100\%} - F_{0\%}} \quad (3.2)$$

Where: $F_{percentage}$: percentage fluorescence (%)

F_w : relative fluorescence of the well of interest (rfu)

$F_{0\%}$: relative fluorescence of the wells with the negative control (rfu)

$F_{100\%}$: relative fluorescence of the wells with the positive control (rfu)

3.9.1.2. Comparison of the MIC results

The MIC of peptide X determined during this optimization assay it is then compared to the MIC of peptide X defined by the control MIC assay described in 3.8.

3.9.2. Cell viability fluorescence screening assay

The black 96-well microtitre plates are inoculated with 0,5-2,5 x 10^5 CFU/mL solutions of the fungal overnight cultures. Further, 30 µL of twelve peptides with an approximate concentration of 150-200 µg/mL are added in respective order in the first row of the plate. A serial dilution of the peptides is conducted, resulting in a one in two dilution series. A one in two serial dilution of the control peptide is carried out as well and a positive and a negative control are included. The control peptide is a peptide with proven activity against gram positive and gram negative bacteria, which is resynthesized in every new peptide library and used to correct for influencing parameters. In the end, 10 µL of the 500 mM resazurin solution is added in every well. This is illustrated in figure 3.2. The sealed plates are incubated at 30°C and are read after 6 hours for *C. krusei* and after 17 hours for *C. neoformans* with a fluorescence microplate reader. This experiment was repeated for all the 670 peptides.

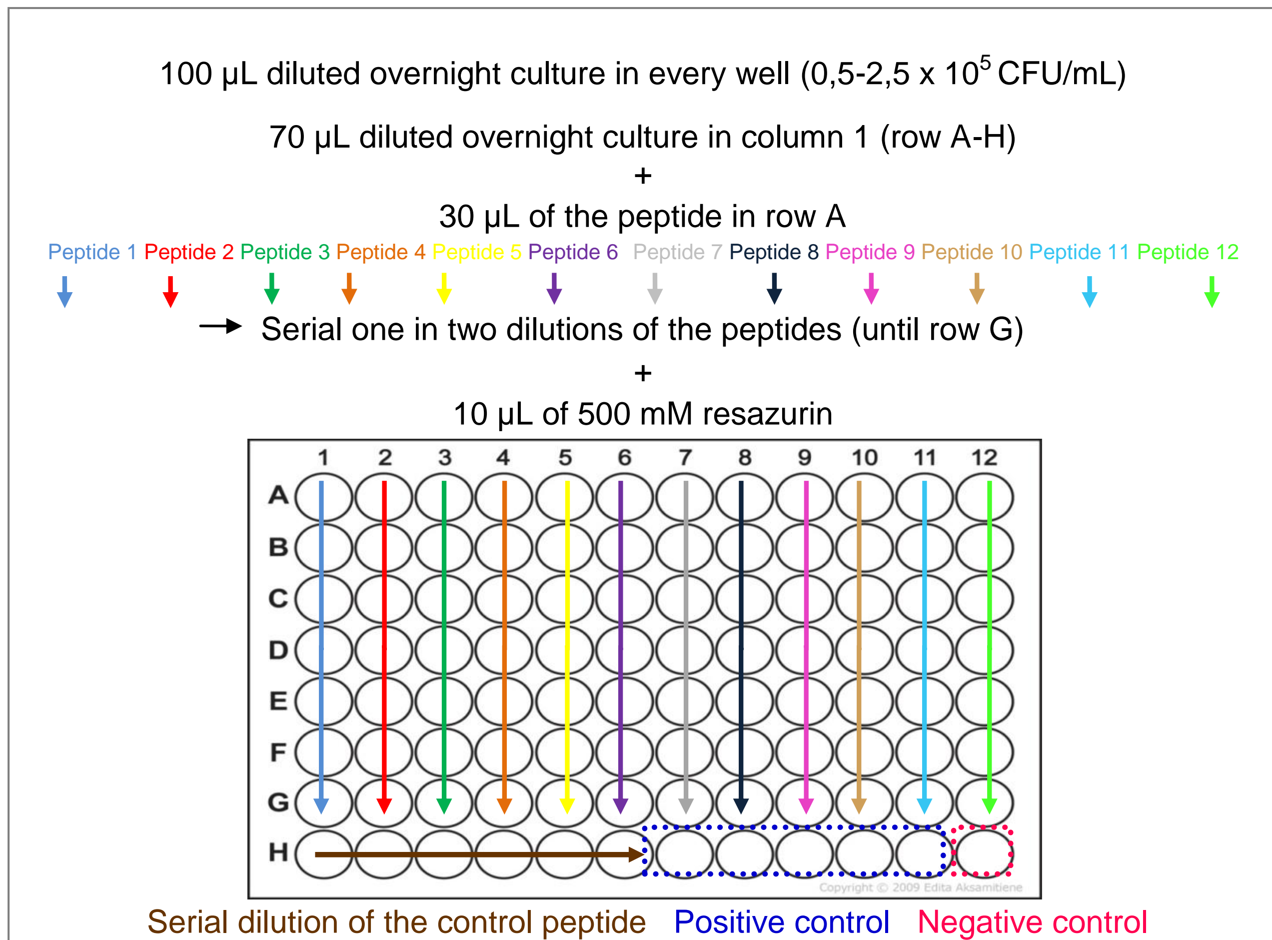


Figure 3.2.: Illustration of the protocol used for the fluorescence screening assay for peptides against *C. krusei* and *C. neoformans.*

The raw data of the fluorescence readings are then analyzed with the Peptide Extension Package of the Matlab Toolbox Gait-CAD software (The MathWorks Inc., Natick, U.S.A.).[39] Bioinformatic models are applied to find a statistical relationship between a peptide sequence or its molecular characteristics and its activity profile.[36] The Gait-CAD software uses fixed values for indicating the peptide concentrations since the exact concentration of the SPOT synthesized peptides are not known. Every next value is divided in two, agreeing with the one in two dilutions used in the screening assay. From the highest until the lowest concentration, we have: 1,00 - 0,500 - 0,250 - 0,125 - 0,063 - 0,031. This is justified because all spots contain approximately the same quantity of peptide.[39]

The peptide activity values are calculated using the 75% inhibitory concentration IC_{75}. The IC_{75} is the concentration of the peptide in which 75% of the fluorescence is inhibited, which correlates with 75% of killing activity of the peptide. It is calculated with formula 3.3, using two concentrations just outlining the IC_{75} and using the logarithm of the activity values A. These concentration are $A(c_1)$ and $A(c_2)$ which are the fluorescence values of the wells immediately after and before the well with 75% inhibition for the peptide which activity is being calculated.[39]

$$IC_{75} = \frac{[\log_{10} A(c_1) - \log_{10} A(IC_{75})] \times c_2 + [\log_{10} A(IC_{75}) - \log_{10} A(c_2)] \times c_1}{\log_{10} A(c_1) - \log_{10} A(c_2)} \quad (3.3)$$

With: $A(IC_{75})$: 25% of the maximum fluorescence for the serial dilution (rfu)

$A(c_1)$:fluorescence in the well immediately after the 75% inhibition well (rfu)

$A(c_2)$:fluorescence in the well immediately before the 75% inhibition well(rfu)

c_1: peptide concentration immediately after the 75% inhibition well (µg/mL)

c_2: peptide concentration immediately before the 75% inhibition well (µg/mL)

The relative IC_{75} is determined by dividing the IC_{75} of the peptide of interest by the IC_{75} of the control peptide on the same 96-well microtitre plate. This results in a more reliable interpretation of the results since any influencing parameter is taking into account and is being normalized for, such as environmental conditions. Using the relative $IC_{75,}$ the peptides are split up into four activity classes. If the relative IC_{75} of the peptide of interest is:[36]

- ≤ 0,5, the peptide is more active than the control peptide and is given the definition 'highly active'.
- 0,5 - 2,0, the peptide has a similar activity to the control peptide and is given the definition 'moderately active'.
- 2,0 - 20, the peptide has a worse activity than the control peptide and is given the definition 'weakly active'.
- ≥ 20, is the peptide inactive and is given the definition 'inactive'.

Due to the high throughput nature of the screening, it is important that the Gait-CAD software identifies any putative errors in the data. These are classified as all peptides where:[39]

- there is an extreme deviation of the control peptide on the plate compared to the median value of the control peptide values of all plates. The IC_{75} value of the control peptide is lower than 50% or higher than 200% of the median IC_{75} value of the control peptides of all plates.
- there is an extreme deviation of the positive control on the plate compared to the median value of the positive control values of all plates. The fluorescence value of the positive control is lower than 25% of the median fluorescence value of the positive controls of all plates.
- there is an unexpected positive correlation between the activity and the peptide concentration, which is expressed as an increase of the fluorescence of at least 30% through the dilution series. It is assumed that as the peptide concentration goes down, the fluorescence value gradually increase through the dilution series.

A further analysis of the results is executed running an analysis of variance (ANOVA) by the Gait-CAD software to distinguish between different relevant features of the peptides in order to characterize their activity. The software scans through more than 500 molecular descriptors, for example the molecular mass, the isoelectric point, the hydrophobicity and so on, and evaluates the mean values of chosen descriptors, the covariance between the four classes and the overall variance of all the results. Preference is given to features which strongly differ in mean values between the four classes and which possess a little covariance within each class. The most promising combination of two descriptors is envisioned in a two-dimensional scatter plot.[39] A complete elucidation of all the algorithms used by the software exceeds the boundaries of this thesis but an overview can be found in the article "Data-Based Activity Analysis and Interpretation of Small Antibacterial Peptides" by R. Mikut and colleagues.[40]

3.10. SYNTHEZING THE SELECTED PEPTIDES ON RESIN

The selected peptides are synthesized on resin using Fmoc-based chemistry. This is kindly performed by the group of Dr. Kai Hilpert. Normally, they are purified up to at least 70% purity by high-performance liquid chromatography (HPLC) and characterized using electrospray ionisation mass spectrometry but this was not possible due to time limitation.

3.11. MINIMAL INHIBITORY CONCENTRATION ASSAY

The broth dilution method is applied following the EUCAST protocol.[33] A concentration of 1 mg/mL of the peptides is prepared. The peptides are diluted down in a one in two serial dilution in 0,5-2,5 x 10^5 CFU/mL solutions of *C. krusei* and *C. neoformans* in a transparent 96-well microtitre plate. A positive and a negative control are also included. The 96-well microtitre plates are incubated for 24 hours at 30°C, after which the results are read visually. This is executed in threefold.

4. RESULTS

4.1. COLONY FORMING UNITS

The CFU/mL is measured three times using formula 3.1 after 24 hours incubation at 30°C of the plates with *C. krusei* and *C. neoformans*. The average of the three measurements is 1,1 x 10^7 for *C. krusei* and 2,0 x 10^7 for *C. neoformans.*

4.2. CONTROLLING THE OVERNIGHT CULTURES

The optical density at 600 nm of the overnight cultures of *C. krusei* and *C. neoformans* is measured after overnight growth in the incubator shaker at 30°C. An overnight culture is prepared every day, which is why the OD is measured several times. The average of these OD readings are 0,27 and 0,22 for *C. krusei* and *C. neoformans* respectively.

The 100-fold diluted concentration of the overnight cultures is determined by counting the colonies on the control plates and using this value in formula 3.1. The graphs showing the correlation between the OD and the log-scale of this concentration can be found in figure 4.1. Using the average OD, the corresponding concentration for *C. krusei*, which is 2,2 x 10^5 CFU/mL, and for *C.* neoformans, which is 2,4 x 10^5 CFU/mL, can be obtained.

According to EUCAST protocol, a final inoculum concentration of approximately 0,5-2,5 x 10^5 CFU/mL is desired. The OD range correlating to this final concentration can be assessed using the graphs in figure 4.1. This range is found to be between 0,10 and 0,28 for *C. krusei* and between 0,05 and 0,22 for *C. neoformans*

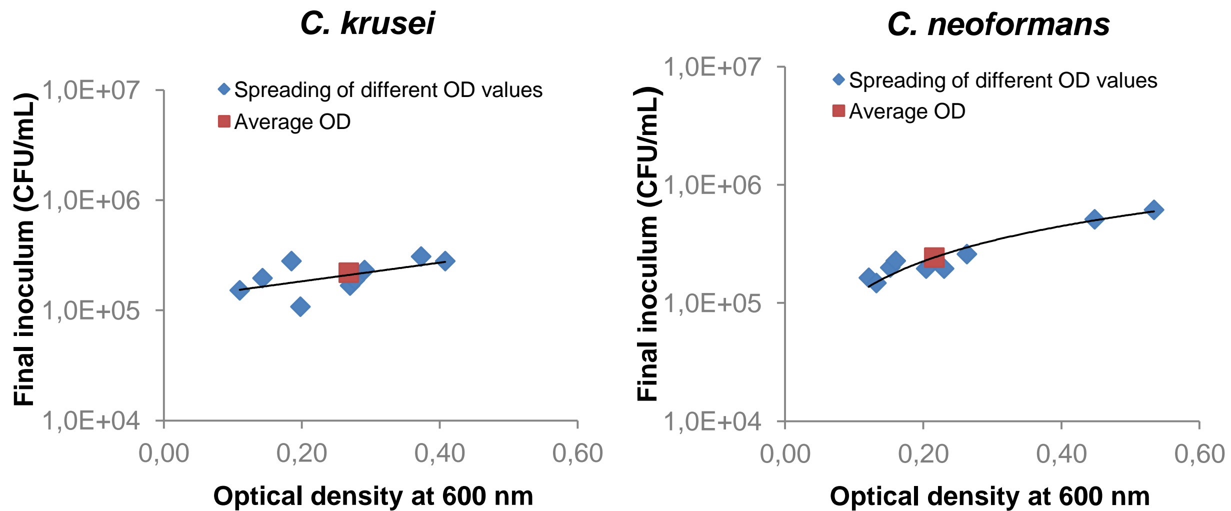


Figure 4.1: Correlation between the optical density at 600 nm and the logarithmic scale of the final inoculum concentration of the overnight cultures determined on the control plates.

4.3. CONTROL MINIMUM INHIBITORY CONCENTRATION ASSAY

This control experiment gives an idea of the susceptibility of both fungi to fluconazole and peptide X. The results are read visually. The MIC is determined as the last well where fungal growth is no longer observed after incubation. Visible sedimentation is regarded as fungal growth. The experiment was repeated three times in order to obtain reliable results. In table 4.1, the MIC of fluconazole and peptide X for both fungi can be found.

Table 4.1.: MIC of fluconazole and peptide X against *C. krusei* and *C. neoformans.*

MIC (µg/mL) determined for:	Fluconazole	Peptide X
C. krusei	62,5	25,0
C. neoformans	/[a]	12,5

[a] MIC against *C. neoformans* is higher than the starting concentration of fluconazole

4.4. CELL VIABILITY FLUORESCENCE SCREENING ASSAY

4.4.1. Optimization of the screening assay

4.4.1.1. Determination of the optimal incubation time point

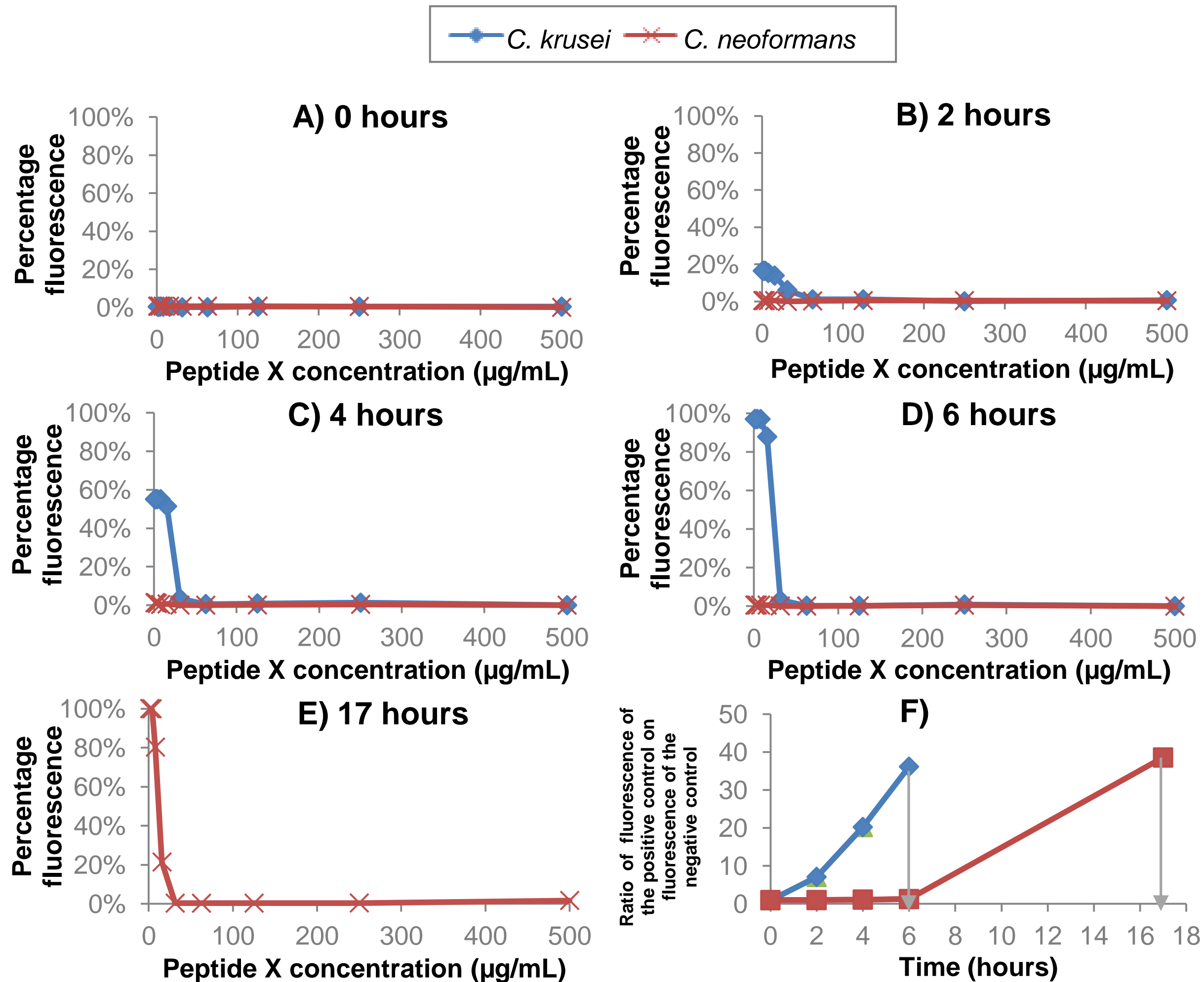


Figure 4.2.: Percentage fluorescence (%) in function of the concentration of peptide X (µg/mL) for the different time points (A-E) and ratio of the fluorescence of the positive control on the fluorescence of the negative control in function of the time (F) to determine the optimal incubation point for *C. krusei* and *C. neoformans* in the resazurin-based screening assay. This optimal incubation time point is 6 hours for *C. krusei* and 17 hours for *C. neoformans.*

In order to identify the optimal incubation time for the fungal strains, the percentage fluorescence is plotted in function of the serial dilution of peptide X for the different time points (graphs A-E in figure 4.2) and the ratio of the relative fluorescence of the positive control (0,5-2,5 x 10^5 CFU/mL fungal cultures) over the relative fluorescence of the negative control (RPMI medium) is plotted in function of the time (graph F in figure 4.2). The percentage fluorescence is calculated using formula 3.2. The optimal incubation time point is 6 hours for *C.* krusei and 17 hours for *C. neoformans.*

4.4.1.2. Comparison of the MIC results

The MIC of peptide X determined in this assay is compared to the MIC of peptide X determined in the control MIC assay. The results can be found in table 4.2.

Table 4.2.: Comparison of the MIC results obtained with the control MIC assay and the resazurin-based fluorescence screening assay.

MIC (µg/mL) determined in:	Control MIC assay	Screening assay[a]
C. krusei	25,0	62,5
C. neoformans	12,5	31,2

[a] using the graph of 6 hours for *C. krusei* and the graph of 17 hours for *C. neoformans* in figure 4.3

4.4.2. Cell viability fluorescence screening assay

4.4.2.1. Activity classification of the peptides

The raw fluorescence data from the screening assay is analyzed using the Gait-CAD software to obtain more information about the peptide characteristics and the relationship to their activity. Gait-CAD firstly identifies the putative errors in the data using the criteria described in the method section. For example, for the data of the synthetic peptides against *C. neoformans*, this quality control identified three peptides that have anomalies which were therefore discounted from the final analysis as artifacts.

Subsequently, Gait-CAD divides the peptides in four classes applying the relative IC_{75} values. The boundaries for this classification can be found in the 3.9.2. The pie charts representing these results can be found in figure 4.3. No synthetic peptides are found to be highly active against *C. krusei*, two synthetic peptides against *C. neoformans*, nine natural peptides against *C. krusei* and five natural peptides against *C. neoformans.*

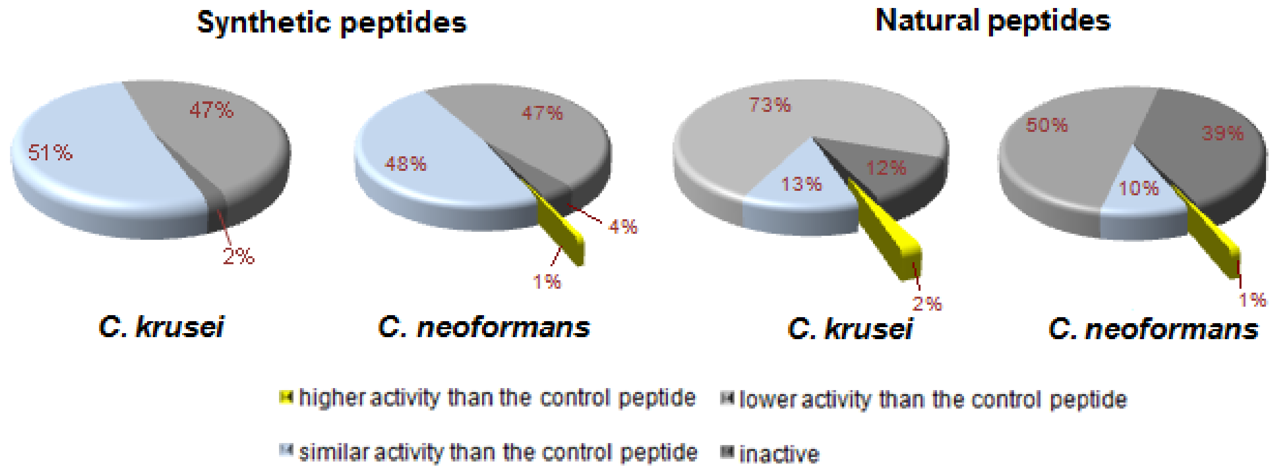


Figure 4.3.: Pie charts splitting up the peptides in four classes based on their relative IC_{75} values. The four classes include peptides which are highly active, moderately active, weakly active and inactive.

The relative IC_{75} values computed by the Gait-CAD software are presented in graphs in which the correlation of the relative IC_{75} of each peptide against *C. krusei* and *C. neoformans* is plotted. The results are presented in figure 4.4.

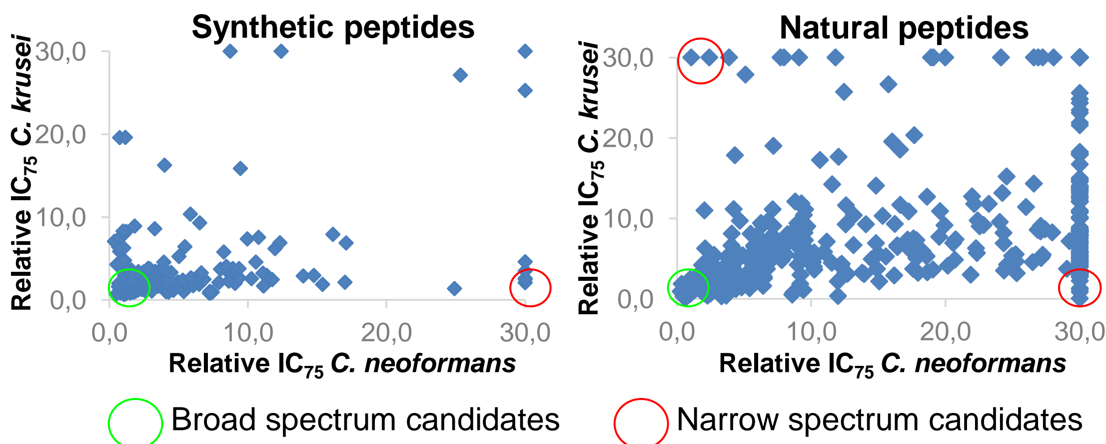


Figure 4.4.: Graphs showing the correlation of the relative IC_{75}s of the synthetic and the natural peptides against *C. krusei* and *C. neoformans.* The correlation coefficients are 0,38 for the synthetic peptides and 0,45 for the natural peptides.

4.4.2.2. Distributional and positional analysis

Gait-CAD verifies if the proportional distribution of the amino acids in the peptide sequences contributes to the level of activity. This is demonstrated in histograms, which can be found in figure 4.5, expressed as the chance of the different amino acids to occur in the sequences. For each amino acid the proportion is split into the four activity classes.

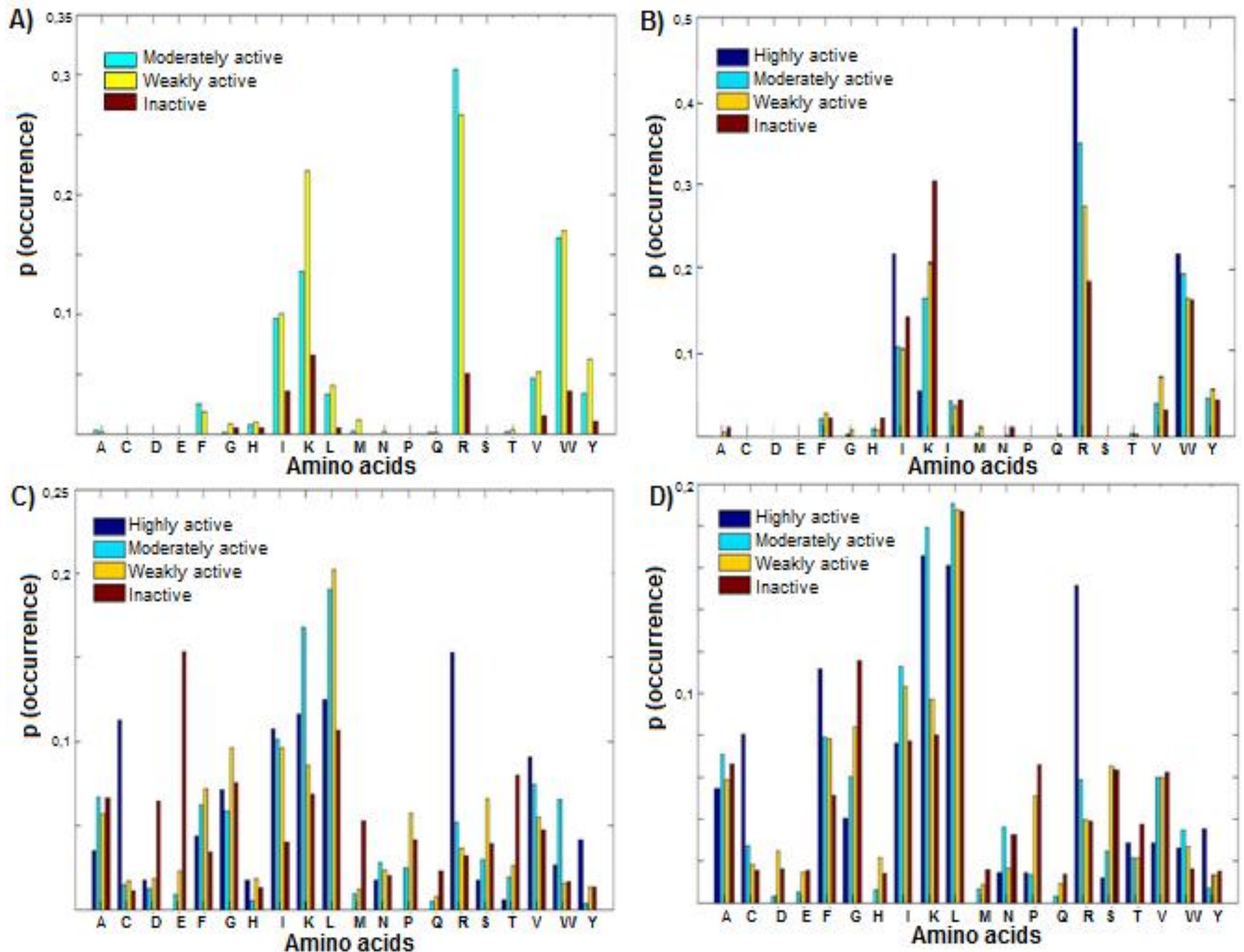


Figure 4.5.: Histogram showing the relative amino acid occurrence in A) the synthetic peptides against *C. krusei,* B) the synthetic peptides against *C. neoformans*, C) the natural peptides against *C. krusei* and D) the natural peptides against *C. neoformans.*

The positional distribution of the amino acids in the peptide sequence is illustrated as the position and the total number of the different amino acids within the peptide sequences, which are subdivided in the four activity classes. This can be found in figure 4.6 for the synthetic peptides. A positional analysis for the natural peptides is not included since all the sequences differ in length.

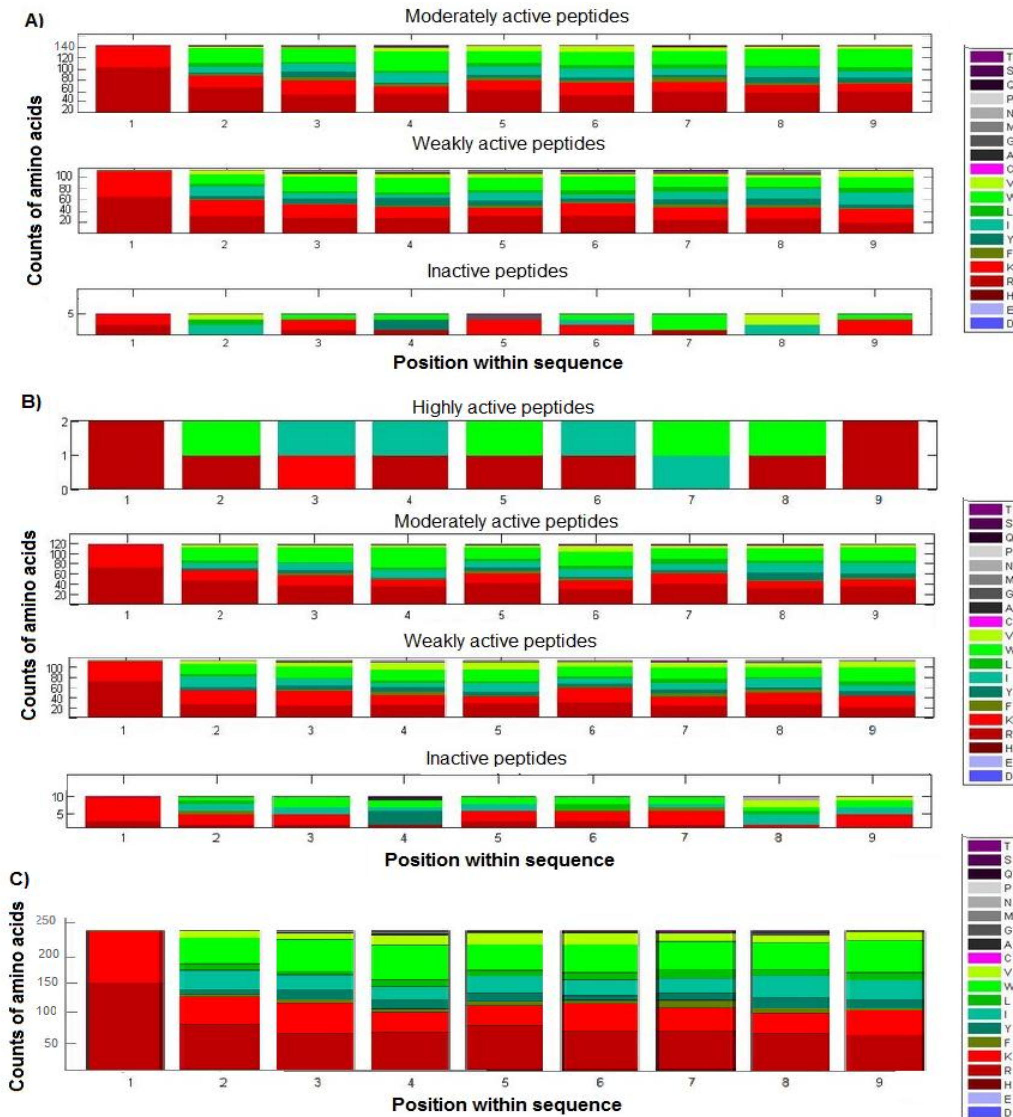
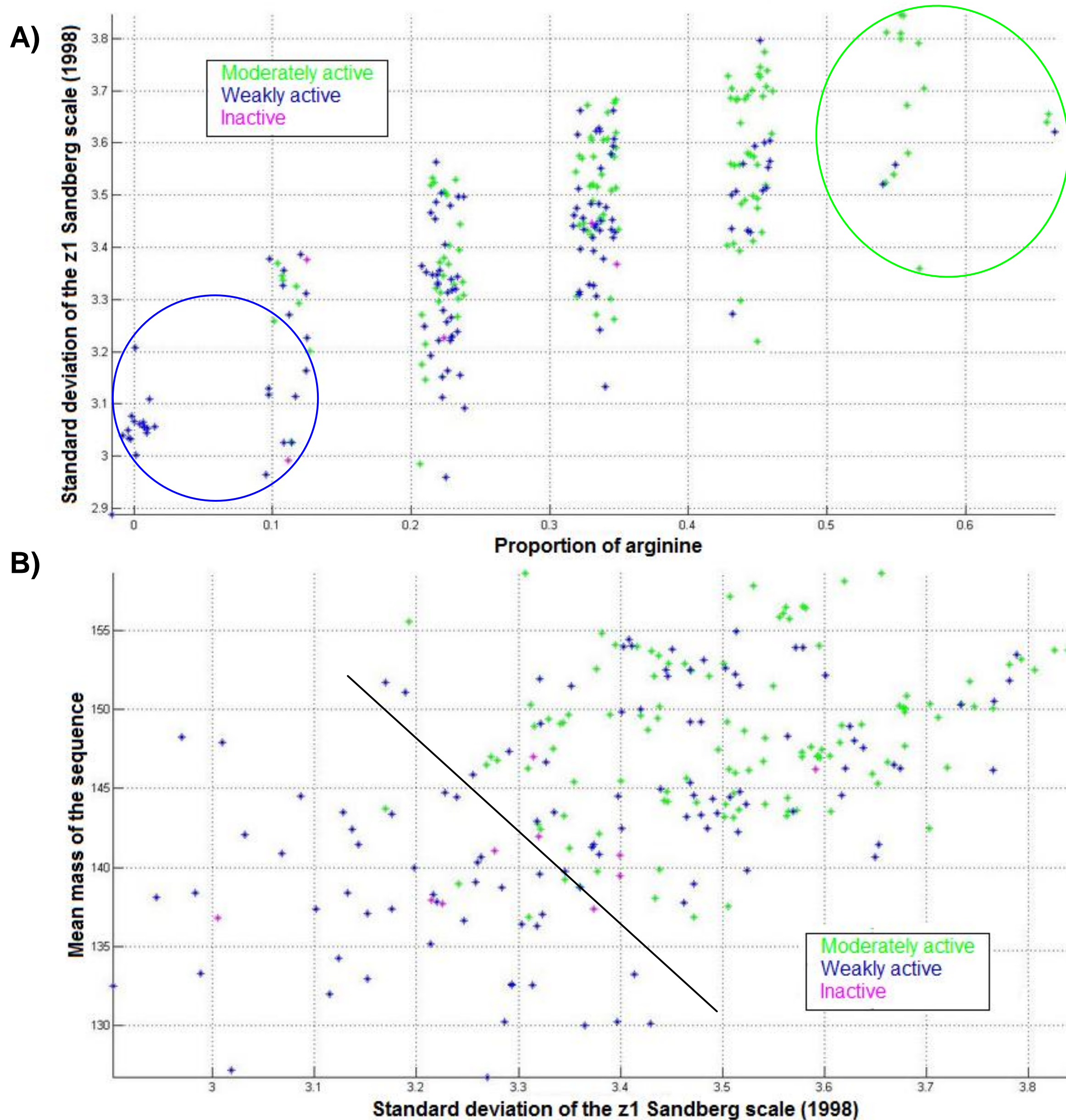


Figure 4.6.: Stacked column chart representing the positional distribution of the amino acids in the synthetic peptides against A) *C. krusei* and B) *C. neoformans*, divided in the four activity classes. Chart C) represents the overall positional distribution of all the synthetic peptides. There are respectively in A) and B) no and 2 highly active peptides, 123 and 116 moderately active peptides, 112 and 112 weakly active peptides and 5 and 10 inactive peptides.

4.4.2.3. Univariate ANOVA analysis

The univariate ANOVA analysis picks the most favorable peptide features based on the criteria discussed in 3.9.2. The scatter plots showing these features can be found in figure 4.7 for the synthetic peptides against both fungi and the natural peptides against *C. krusei*. The peptides in the highly activity group are excluded due to the low number of peptides in that group which can influence the choice of descriptors. The scatter plot for the natural peptides against *C. neoformans* did not show any remarkable trend and was therefore not included.



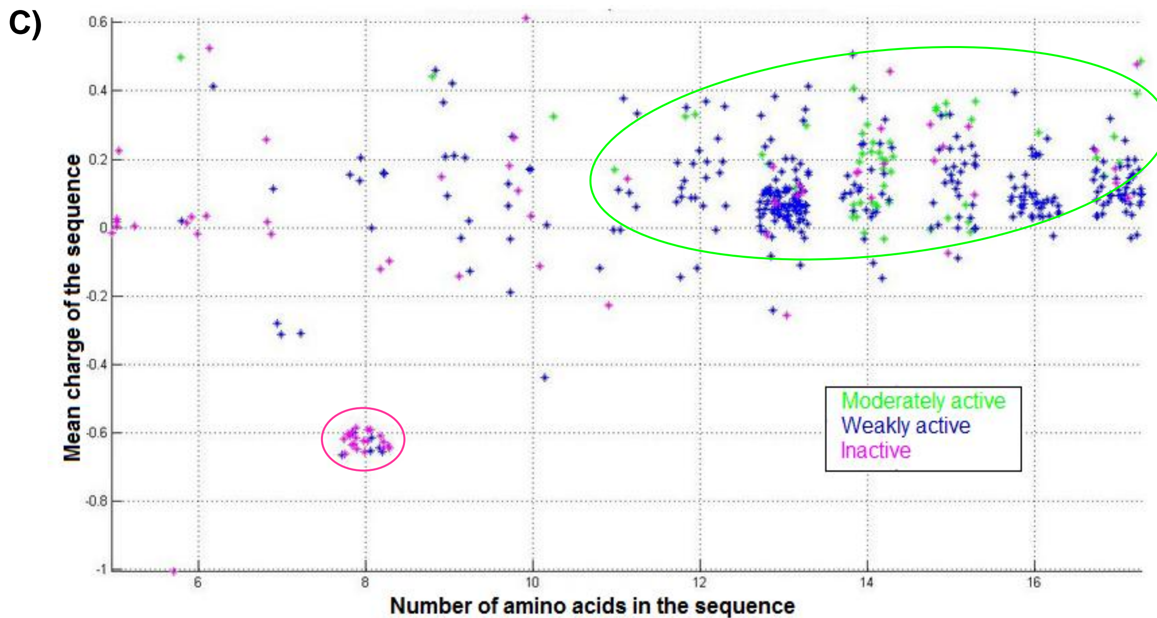


Figure 4.7.: Two-dimensional scatter plots visualizing the univariate ANOVA analysis of the Gait-CAD selected features for the activity classes, with exclusion of the highly active activity class, of the peptides. Respectively for A) the synthetic peptides against *C. krusei*, B) the synthetic peptides against *C. neoformans* and C) the natural peptides against *C. krusei*.

4.5. MINIMAL INHIBITORY CONCENTRATION ASSAY

Due to time limitation a purification step could not be included, which is why only the peptides with an acceptable purity (>70%) after synthesis on resin could be tested for their MIC. These were only peptide 380 and peptide 461. The MIC results can be found in table 4.3.

Table 4.3.: MIC results of the synthetic peptides 380 and 461 against *C. krusei* and *C. neoformans*

MIC (µg/mL) determined for:	Peptide 380	Peptide 461
C. krusei	62,5	31,3
C. neoformans	15,6	15,6

5. DISCUSSION

5.1. COLONY FORMING UNITS

The CFU is calculated in order to estimate which dilution of the overnight culture needs to be made to achieve the EUCAST-recommended final inoculum of 0,5-2,5 x 10^5 CFU/mL.[33] Since a result of 1,1 x 10^7 CFU/mL for *C. krusei* and 2,0 x 10^7 for *C. neoformans* is obtained, the decision was made to perform a 100-fold dilution of the overnight cultures.

5.2. CONTROLLING THE OVERNIGHT CULTURES

Because all the OD values measured were fairly deviating from each other, an average of the ODs was taken to verify if the 100-fold dilution of the overnight cultures has the demanded final inoculum concentration. These values are 2,2 x 10^5 for *C. krusei* and 2,4 x 10^5 for *C. neoformans* and are therefore close to exceeding the limits defined by EUCAST.[33] A higher inoculum can deteriorate the results for the peptides, while a smaller inoculum can lead to false positive results and thus to an overestimation of the peptide activity.[34] The OD values are depending on many variables, for example the growth of the colonies on the streak plate, the temperature fluctuations in the incubator shaker during the night and so on. The OD is only used as a rough estimation to verify if the value of CFU/mL in the overnight cultures is in the desired range. The control plates after an assay are there to confirm if the 100-fold diluted starting concentration is still in the correct range and therefore if the OD reading before was accurate.

5.3. CONTROL MINIMUM INHIBITORY CONCENTRATION ASSAY

The MIC can be used to classify the fungi as susceptible, intermediate and resistant to the investigated drug. The EUCAST defines these breakpoints for *Candida* spp. *Candida* spp. are defined resistant to fluconazole if the MIC value exceeds 64 µg/mL. Since the MIC result for the tested *C. krusei* strain is found to be 62,5 µg/mL, the strain would normally be considered in the intermediate susceptibility category. However, *C. krusei* is an exception in the *Candida* spp. because it is intrinsically resistant to fluconazole, which is why susceptibility testing for fluconazole against *C. krusei* is not recommended.[33,41]

There was sedimentation in every well in the 96-well microtitre plates containing *C. neoformans* and fluconazole. This means that the MIC of fluconazole against *C. neoformans* is higher than the starting concentration of 125 µg/mL. This is higher than the normal MIC range determined in previous studies.[23] Therefore, the conclusion can be made that the used *C. neoformans* strain is resistant to fluconazole.

Now is the question if it is acceptable to choose a drug to which the tested strains are resistant as a control. Fluconazole is mainly used as verification if the used strains are acting as expected. However, peptide X is used in the following experiments. It is a lab stock peptide with proven activity against both tested fungi. The MIC determining EUCAST method is confirmed to have significant reproducibility and reliability for fungal susceptibility testing.[33] The MIC results of peptide X are discussed in 5.4.1.

5.4. CELL VIABILITY FLUORESCENCE SCREENING ASSAY

5.4.1. Optimization of the screening assay

5.4.1.1. Determination of the optimal incubation time point

The optimal incubation time point can be identified using the graphs in figure 4.2. The peptide needs to be able to execute its activity against the fungal cells during the period of incubation. This is verified by the percentage fluorescence calculated using formula 3.2. The highest peptide X concentration needs to kill all fungal cells, which is displayed by a 0% fluorescence which corresponds to the percentage fluorescence of the negative control or the RPMI medium. This means that no growth is observed. While going to a lower concentration of peptide X, a gradual increase in the percentage fluorescence is observed, which indicates that a lower number of fungal cells is killed. The concentration of peptide X is decreased in the dilution series and at the lowest concentration, a 100% percentage fluorescence is detected. This corresponds to the same percentage fluorescence as the positive control, which means that the fungal cells have developed to full growth. The graphs A-E representing this in figure 4.2, illustrate that the 100% percentage fluorescence for the lowest peptide X concentration is obtained at 6 hours for *C. krusei* and at 17 hours for *C. neoformans.*

Another requirement is a high ratio of the rfu of the positive control and the rfu of the negative control in order to become a high signal to background ratio. This also means that the difference in rfu of the viable cells compared to the wells with no growth is approximately at its highest level. Furthermore, it verifies if resazurin had enough time to enter the fungal cells, where it is whether reduced by the viable cells or not. This is possible since only the positive and negative control results are included in the graph. Graph F representing this in figure 4.2 confirms that the optimal incubation time for *C. krusei* is again around 6 hours and for *C. neoformans* around 17 hours. If the strains are incubated for longer, there is a possibility that they reach death phase in the growth curve which can influence the accuracy of the results.

5.4.1.2. Comparison of the MIC results

For the comparison of the MIC results of peptide X, it is clear that the MIC results determined in the resazurin-based screening assay are higher than those determined in the control MIC assay, which is expected since the screening assay is a more sensitive technique because the fluorescence readings are quantified using a sensor. This leaves less room for interpretation, contrary to the control MIC assay, in which the results are read visually. There is a possibility that growth in the well cannot be determined with the naked eye but can be stipulated using fluorescent readings. However, a correlation can be observed between the two MIC-determining techniques.

5.4.2. Cell viability fluorescence screening assay

5.4.2.1. Activity classification of the peptides

The pie charts in figure 4.3 represent the distribution of the peptides in the four activity classes. There are no synthetic peptides highly active against *C. krusei* due to the fairly strong activity of the control peptide against *C. krusei*. The relative IC_{75} values are therefore elevated. The control peptide is a peptide with a proven activity against gram positive and gram negative bacteria. The same sequence for this peptide is used for the synthetic and the natural peptides but they are produced in different synthesises.

Therefore, the power of the killing activitiy can slightly differ depending on different parameters correlating to the synthesis, such as the purity, the quantity and so on. Another remarkable fact is that 51% of the synthetic peptides are highly or moderately active against *C. krusei* and 49% against *C. neoformans*, compared to 15% for the natural peptides against *C. krusei* and 11% against *C. neoformans.* This is because the synthetic peptides already have been optimized for their activity against *P. auruginosa* and these modifications could also have an effect on the activity against both tested fungi. It is interesting to know that those optimizations possibly also resulted in a broader spectrum activity.

The first graph in figure 4.4 shows a light trend of correlation between the antifungal activity against *C. krusei* and *C. neoformans* for the synthetic peptides, with a correlation coefficient of 0,38. Additionally, the graph indicates that there are some broader spectrum candidate peptides against both fungi and also a few selective candidates against *C. krusei.* On the other hand, there is a slightly positive trend observed in the second graph between the antifungal activity of the natural peptides against both fungi, with a correlation coefficient of 0,45. There is also a considerable number of broad spectrum candidates for the natural peptides and a few selective peptides against *C. krusei* and *C. neoformans.* The Gait-CAD software numbers the inactive peptides from the value 20 to 100 for their relative IC_{75}, which makes the spread for in the inactive group fairly broad, comparing to the peptides in the other three classes. To account for this, every relative IC_{75} over 30 is changed to the number 30.

5.4.2.2. Best peptides and their broad spectrum activity

The most active peptides against *C. krusei* and *C. neoformans* are represented in table 5.1. They are classified using the relative IC_{75}s and boundaries stated in 3.9.2. The sequences are abbreviated with the commonly used one-letter symbols approved by the International Union of Biochemistry and the International Union of Pure and Applied Chemistry (IUB-IUPAC).[42] The activity classification for these peptides against *Candida albicans* (*C. albicans*)*,* MRSA and *P. aeruginosa* is also included in table 5.1. These results are kindly provided by the lab group of Dr. Kai Hilpert but are not published yet.

Table 5.1.: Comparison of the activity of the best peptides against *C. krusei* and *C. neoformans* with their activity against *C. albicans*, MRSA and *P. aeruginosa*.

Highly active | Moderately active, tending to highly active | Moderately active | Weakly active | Inactive

Peptide number	Sequence	*C. krusei*	*C. neoformans*	*C. albicans*[a]	*MRSA*	*P. aeruginosa*[a]
Synthetic peptides						
419	KKRLRWIRW					
461	RRVKIRVYW					
525	RWKIRIIWR					
559	RRIRWRWRR					
Control	KRRWRIWLV					
Natural peptides						
59	KKLLKWLKKLL					
77	RICRIIFLRVCR					
221	KKKKPLFGLFFGLF					
257	AILTTLANWARKFL					
312	VNWKKILGKIIKVVK					No data
328	RGGRLCYCRRRFCICV					
367	GLFDIVKKIAGHIASSI					
373	INIKDILAKLVKVLGHV					
374	INVLGILGLLGKALSHL					
375	KWCFRVCYRGICYRKCR					
376	KWCFRVCYRGICYRRCR					
377	RWCFRVCYRGICYRKCR					
378	GVLDAFRKIATVVKNVV					
391	ALYKKFKKKLLKSLKRL					
Control	KRRWRIWLV					

[a] interpretation of the activity classes without use of Gait-CAD software

Peptides 461 and 525 are the synthetic peptides with a broad spectrum activity. Peptide 461 has a moderate activity against both tested fungi and peptide 525 against *C. krusei* but the relative IC_{75}s are close to the border separating the highly active from the moderately active group, which is a relative IC_{75} of 0,5. The peptides can be considered as tending to be highly active since the activity classification is defined by very precise borders for a large range of different relative IC_{75}s. For example, a peptide with a relative IC_{75} of 0,51 will be considered moderately active while a peptide with a relative IC_{75} of 0,49 will be considered highly active. Further, previous studies have shown that peptides with arginine in position one and hydrophobic amino acids in position five, seven and nine tend to have a higher antimicrobial activity.[43] This is found in peptide 461 and partially in peptide 525. It is also interesting to find some narrow fungal spectrum peptides. This is found in peptide 419, which is only moderately active against *C. krusei*. Another selective peptide is peptide 559, which is highly active against *C. neoformans.*

The natural peptides 59, 257, 375, 376 and 377 are considered as broad spectrum peptides. Peptide 59 or L5K5W from the LK-peptide class is already proven to have a gram positive and gram negative spectrum activity and a low hemolytic activity. The use of only leucines and lysines with one tryptohan between the hydrophilic lysine and the hydrophobic leucine results in an amphipathic helical peptide, which can result in antimicrobial killing potential.[10,44] The cationic peptide 257 is highly active against *C. neoformans* and moderately active against the other tested organisms. It is named temporin-HN1 and is found in the frog *Odorrana hainanensis.* It is proven to be active against gram positive and some gram negative bacteria and fungi. Its structure is mainly α-helical. It showed a low hemolytic activity against human erythrocytes *in vitro*.[10,45]

Peptide 375, 376 and 377 are respectively tachyplesin III, tachyplesin I and tachyplesin II, found in horseshoe crabs. Tachyplesins are 17 to 18 amino acids long cationic AMPs. Three tandem repeats of a sequence consisting of an hydrophobic amino acid, a cysteine residue, an aromatic amino acid and arginine can be found in the three peptides.[10,45] This indicates that an amphipathic structure is a cooperating to its activity. The glycine on the tenth position is also present in the three sequences.

A Nuclear Magnetic Resonance (NMR) study has shown that tachyplesins have a beta hairpin secondary structure with the glycine residue in the middle of the loop, which is therefore critical for the conservation of the secondary structure of the peptides.[46,47] They have an activity against gram positive and some gram negative microorganisms. Tachyplesin I has an extra activity against viruses, such as HIV, and cancer cells.[10]

The fungal selective peptides are peptides 77, 367 and 378. Peptide 77 is highly active against *C. krusei* and *C. neoformans* but weakly active against *C. albicans*, the gram positive MRSA and the gram negative *P. aeruginosa.* This is a cysteine-rich cyclic docapeptide named bactenecin-1, belonging to the AMP family of the cathelicidins and derived from sheep. It is predicted to target some gram positive and gram negative bacteria, such as *Straphylococcus aureus* and *Escheria coli*. Bactenecin, which is also a cyclic docapeptide but derived from bovines, is already been proven to have an antimicrobial activity against gram negative bacteria. Its sequence differs from bactenecin-1 with a leucine on position two, two valines on positions six and seven and an isoleucine on position eight. They both have one disulfide bond.[10,48]

Peptide 367 en peptide 378 show only activity against *C. krusei* and can therefore be used as selective agents against infections caused by *C. krusei*. In previous studies, it has been proven that peptide 367 has activity against some gram positive and negative bacteria, against fungi and against cancer cells. MRSA, *C. albicans* and *P. aeruginosa* were not included in these test results.[49] Its name is aurein 3.2 and it is originating from the Australian frog *Litoria aurea* or *Litoria raniformis.*[10] Peptide 378 or uperin 3.1 from the Australian floodplain toadlet *Uperoleia inundata* is also proven to have activity against some gram positive and gram negative bacteria.[10] When comparing the peptide sequences of 367 and 378, some similarities can be noticed which can therefore maybe be used for optimization to obtain *C. krusei* selective peptides. They are 17 amino acids in length and have a glycine on position one, an aspartic acid on position four and the tandem lysine-isoleucine-alanine on positions eight till ten. It has been proven that the hydrophilic aspartic acid on position four does not contribute to the antimicrobial activity but the hydrophilic lysine on position eight does.[49]

5.4.2.3. Distributional and positional analysis

The histograms in figure 4.5 represent the distribution of the different amino acids within the sequences in the four activity classes and the relative amino acid occurrence expressed as a p-value. Firstly, the synthetic peptides are discussed. The histograms A) and B) for the synthetic peptides against *C. krusei* and *C. neoformans* show an increase of activity with an increase in the arginine content. The fungal killing potential is primarily associated with a higher number of tryptophan for *C. neoformans*. The importance of the positively charged arginine and the hydrophobic tryptophan for the antimicrobial activity has been proven in various studies. It has been confirmed that a balance between the hydrophobicity and the positive charge needs to be maintained to achieve an optimal antimicrobial activity.[50] The association of arginine with tryptophan in the peptides shields arginine from the hydrophobic environment in the cell wall or membrane.[51] Arginine provides a positive charge and a donor hydrogen bond, which are crucial for the interaction with the negatively charged components on the fungal cell wall and the bacterial cell membrane.[51] The predominance of both amino acids in AMPs results in an increase in the activity.[51,52]

Further, the right balance is required for lysine in the synthetic peptides against *C. krusei*. Too much lysine results in weakly active peptides but not enough lysine ends in inactive peptides. The same goes for leucine and tyrosine but to a lesser extent. In the case of *C. neoformans*, more lysine leads to worse activity. This can be explained by the understanding that both arginine and lysine are able to form cationic interactions with the negatively charged fungal cells; however, only arginine has the capability to form hydrogen bonds both when involved in a cationic interaction. These cationic interactions make it energetically less challenging for the arginine residue to enter the hydrophobic lipid bilayer area. Since lysine is not able to do both interactions (hydrogen bond and cationic interaction), a preference is given to peptides in which arginine instead of lysine is substituted.[51]

An example of the lysine influence on the activity of the peptides can be given when looking at the relative IC_{75} data and comparing similar sequences which mostly differ in their lysine content. It can be noticed that the sequence with three lysine residues possesses a weak activity against *C. krusei* and no activity against *C. neoformans.* Two lysine residues in a comparable sequence results in a weakly active peptide against *C. krusei* and *C. neoformans*, so the activity is already increasing against *C. neoformans* when the lysine content decreases. One lysine residue can lead to both moderately, weakly active and inactive peptides against *C. krusei* in similar sequences. This contributes to the statement before that the right balance in lysine is required, also correlating with the arginine content. For *C. neoformans*, one lysine residue in the comparable sequences gives both moderately and weakly active peptides, which also shows that a lower lysine content ends up in stronger peptides against *C. neoformans.*

The stacked column charts in figure 4.6 represent the distribution of the amino acids in the different positions of the peptide sequences linked to one of the four activity classes. The individual column charts (figure 4.6, A and B) are compared to the overall positional analysis of all the synthetic peptides (figure 4.6, C). The chart for the synthetic peptides against *C. krusei* (figure 4.6, A) indicates that a higher proportion of arginine correlating with a lower lysine content on all the positions results in improved activity. Previous data analysis showed that a higher arginine and a lower lysine content were associated with a higher antimicrobial activity and were therefore used in the optimization of these peptides.[42] Other trends observed are less consistent through all the positions and need to be examined with more caution. These trends mainly apply to the hydrophobic amino acids isoleucine, leucine, tryptophan and valine, which are abundant in the synthetic peptides. A higher proportion of these amino acids is located on position four of the moderately active synthetic peptides against *C. krusei*, except for valine of which a slightly lower amount is found in these peptides. A lower prevalence of these hydrophobic amino acids on position nine also results in stronger peptides against *C.* krusei, except for tryptophan of which a higher amount is desired. A former study proved that hydrophobic amino acids at the amino terminus are more crucial for the antifungal activity than at the carboxyl terminus.[53]

On the other positions is both a increasing or decreasing trend observed alternately for these hydrophobic amino acids in the moderately active synthetic peptides against *C. krusei*. An increase in the hydrophobic phenylalanine on positions three and four and a decrease of tyrosine on positions two and six also results in a better activity against *C. krusei.* The synthetic peptides chart for *C. neoformans* (figure 4.6, B) shows more or less the same trends as the column chart for *C. krusei* when comparing the moderately active group with the weakly active group but they are less significant. There is a more outspoken decrease in valine on positions two, three, four and five correlating to a higher activity.

The previous assumptions are all made comparing the data of the moderately active group with the weakly active group because only enough and approximately equally as much synthetic peptides are included in these groups (123 peptides moderately active and 112 weakly active comparing to 0 active and 5 inactive for *C. krusei* and 116 peptides moderately active and 112 weakly active comparing to 2 active and 10 inactive for *C. neoformans*). The trends observable in the inactive peptide group for *C. krusei* are that almost no arginine is present anymore and that this is mainly substituted for lysine on positions one, three, five, six and nine. However, positions four and seven only contain arginine and no lysine. For *C. neoformans,* the two active peptides both have arginine in position one and nine. For the inactive group, there is more lysine compared to arginine on position one and a high number of tyrosine, which is also proven before in studies to have negative effect on the activity,[54] and almost no positively charged amino acids on position four. The previous presumptions need to be perceived with caution since not enough results are included to make an accurate conclusion.

The natural peptides can only be examined by an analysis of the distribution of the amino acids since a positional analysis is not possible due to the variation in length of the sequences. It also has to be taken into account that the natural peptides differ sufficiently in sequence composition comparing to the synthetic peptides, wherefore an analysis of all the natural peptides should be taken with more caution. These are the most remarkable trends for both fungi showed in histograms C and D in figure 4.5:

more of the positively charged arginine and lysine and the hydrophobic isoleucine and tryptophan leads to a higher activity and more of the negatively charged glutamic acid and aspartic acid (mainly for *C. krusei*) and more of glutamine and methionine to a lower activity. Glutamic acid and aspartic acid are anionic amino acids and are therefore less able to interact with the also negatively charged components in the fungal cell walls.[51] Methionine has a sulfur atom which can easily be oxidized, which results in destabilization of the peptide and its activity.[51]

Furthermore, it can be noticed that for the activity against *C. krusei* less of the hydrophilic threonine, more of the hydrophobic valine and the right balance for phenylalanine, proline, serine and leucine is desired. This can be found in the selective *C. krusei* peptides 328 (no threonine, one valine, one leucine), 367 (no threonine, one valine, one phenylalanine, two serines, one leucine), 373 (no threonine, three valines, three leucines) and 378 (one threonine, five valines, one phenylalanine, one leucine). Interestingly, a higher activity for *C. neoformans* is observed if more cysteine and less glycine and serine are inserted in the sequence. Cysteine and sometimes glycine can bind to the chitin in the fungal cell walls and therefore contribute to a higher activity.[55] For this analysis, the moderately active, weakly active and inactive groups are compared since the highly active groups do not contain enough results (55 moderately active, 313 weakly active and 52 inactive peptides versus 10 active peptides for *C. krusei* and 43 moderately active, 214 weakly active and 168 inactive peptides versus 5 active peptides for *C. neoformans*).

5.4.2.4. Univariate ANOVA analysis

The univariate ANOVA analysis leads to different scatter plots with various descriptors best matching the peptide sequence. These are represented in figure 4.7. This bioinformatic approach is used to convert the peptide sequences into valuable descriptors which can be associated to the activity profile. The highly active peptide group is excluded out of all the ANOVA plots since the low proportion of peptides in these groups can result in incorrect selection of the best features and misinterpretation of the results.

For the synthetic peptides against *C. krusei* (figure 4.7, A), the proportion of arginine and the standard deviation of the Sandberg z1-scale established in 1998 are chosen. The proportion of arginine correlating with a higher activity is already proven by preceding data and earlier studies.[43] The Sandberg scale is an experimentally determined scale established by Sandberg *et al.* It uses 87 amino acids distinguished by 26 physicochemical characteristics, such as molecular mass, retention time obtained by thin-layer chromatography, side chain charge, van der Waals volume of the side chain and so on. These descriptors are represented in five z-scales (z1 until z5) of which the z1-scale is applied for this scatter plot. The z1-scale can be interpret as a hydrophobicity estimation.[56] Since the standard deviation is used and a slightly positive trend is observed with the activity, high proportions of highly hydrophilic and highly hydrophobic amino acids can lead to an increase in activity against *C. krusei.* This can be confirmed by the previous data (figure 4.6), showing that a high number of the hydrophobic amino acids such as tryptophan, leucine, valine and isoleucine on certain positions and the hydrophilic arginine resulted in a higher activity. The standard deviation of the Sandberg z1-scale is also selected for the peptides against *C. neoformans* (figure 4.7, B). This is also confirmed in the previous results, with lysine as an exception leading to a decrease in activity. The other feature chosen for *C. neoformans* is the mean mass of the amino acids in the peptide sequence. Tryptophan as the heaviest amino acid but also the heavy arginine are correlated to a higher activity.

The scatter plot for the natural peptides against *C. krusei* in figure 4.6, C shows that a higher number of amino acids in the sequence is associated with a higher activity. The peptides with the highest activity are more around 10 to 17 amino acids in length. Preference is mostly given to shorter peptides since they are synthesized, modified and optimized on a user-friendlier, faster and more economical scale. Longer peptides seem to be harder to synthesize and the results are more challenging to interpret. Moreover, the purity of longer sequences is lower compared to shorter ones in most cases.[57] A good balance between the best activity and the advantages of using shorter peptide sequences needs to be found.

The other descriptor is the mean charge of the sequence. There is a remarkable group of outliers of weakly active and inactive peptides with a negative mean charge. It has been proven that anionic peptides possess a rather weak antimicrobial activity, compared to cationic peptides.[57] The cationic peptides do not seem to show a significant trend. Most of these peptides seem to have a mean charge between 0,0 and 0,4. The scatter plot for the natural peptides against *C. neoformans* did not show any interesting trends and is therefore not included.

5.4.3. Limitations

A few things should be taken in consideration when reading the results of the screening assay. Firstly, because the preparation steps are completed manually, errors can occur unnoticed. Another limitation for fluorescence readings is the background noise which can be induced by several causes, such as autofluorescence coming from components different from the fluorophore of interest.[58] This is partially circumvented by using black 96-well microtitre plates instead of transparent ones. Transparent or white plates will reflect the light maximally due to its white color and therefore increase background fluorescence, while black ones extinguish the light and reduce the signal.[59] The autofluorescence of the medium is verified by including a negative control in the assay.

Antimicrobial peptides are still somehow unpredictable in some aspects, such as degradation processes. These can influence the results and need to be taken into account when testing the peptides in a further stage.[60] Also, not all the molecular descriptors resulting in a more active peptide are known at this point. Future investigation for this part is necessary.[60] Since the peptides are synthesized using SPOT synthesis, their concentration is not defined exactly. A peptide can therefore show a false positive or negative result when a really high or low concentration is synthesized.

5.5. MINIMAL INHIBITORY CONCENTRATION ASSAY

Both peptides tested are not the most interesting ones of all the peptides synthesized on resin but only these two had a purity which was high enough to start the MIC determination without performing a purification step. A purification step was not possible to carry out due to time limitation. Both peptides are moderately active with peptide 461 tending to be highly active (mainly against *C. neoformans*). This is visible in the MIC results for peptide 461 with an MIC of 31,3 µg/mL against *C. krusei* and of 15,6 µg/mL against *C. neoformans.* Peptide 380 shows the same MIC result against *C. neoformans* as peptide 461, but a higher MIC against *C. krusei*, in particular 62,5 µg/mL.

Voriconazole is often used as an alternative treatment against *C. krusei,* since *C. krusei* is naturally resistant to fluconazole. The MIC for voriconazole is normally in the range of 0,25 and 0,50 µg/mL.[61] There are various antifungal agents available against *C. neoformans*, depending on disease and patient criteria. Here, also voriconazole will be compared to have a more equally comparison with *C. krusei.* A range of 0,03 and 0,25 µg/mL is normally found for this agent against *C. neoformans.*[62] When comparing these MICs with the MICs of the tested peptides, it indicates that the currently used drugs still have a lower MIC. It must be taken into account that these peptides are not most active ones that could be tested and that they still need to be optimized against both fungi.

5.6. TIME LIMITATION AND FUTURE WORK

The biggest limitation of this project was time. Due to this not all highly active peptides could be synthesized on resin and tested for their MIC. The time pressure also made it impossible to include a quality control of the peptides synthesized on resin. All these steps mentioned are the first things that have to be done next. One of the following steps is to control the hemolytic activity of the peptides by a hemolysis screen on human erythrocytes.

Another future plan is to optimize both peptides libraries for their activity against both fungi. The previous analysis results can assist in this step. Since the synthetic peptides are already optimized against *P. aeruginosa*, further optimization beneficially for *C. krusei* and *C. neoformans* can give hopefully broader spectrum peptides or alternatively can give selective peptides towards fungi. One of the possibilities to do this is a substitutional analysis where each position in the peptide sequences can be exchanged by all the twenty L or D amino acids. It is to determine which substitution at a certain position of the peptides improves its activity. Then, the most promising substitutions can be combined into new optimized peptide sequences.[63] Another method is the generation of combination peptides, for example combining one of the peptides with a polysaccharide or with a lipid.[64]

A next step would be to test the chosen antimicrobial peptides in animal models. For example, zebrafishes which can be used for toxicity studies[65] or mice used as an infection model or to study pharmacokinetics and pharmacodynamics of the drugs. After, the peptide can proceed to clinical trials and become officially registered as a drug.

6. CONCLUSION

The main objective of this research was to find interesting peptide candidates in the two peptide libraries against *C. krusei* and *C. neoformans*. There were seven highly active peptides found against *C. neoformans*, of which two are from the synthetic peptide library (525 and 559) and five are from the natural peptide library (77, 221, 257, 376 and 391). Nine peptides of the natural peptide library were identified as highly active against *C. krusei* (59, 77, 328, 367, 373, 375, 376, 377 and 378), while none of the synthetic peptides are. Both selective peptides against *C. krusei* (the synthetic peptide 419 and the natural peptides 77 or bactenecin-1, 367 or aurein 3.2 and 378 or uperin 3.1) or against *C. neoformans* (the synthetic peptide 559 and the natural peptide 77 or bactenecin-1) and broad spectrum peptides (the synthetic peptides 461 and 525 and the natural peptides 59 or L5K5W, 257 or temporin-HN1 and the tachyplesins or peptide numbers 375, 376 and 377) were detected.

Subsequently, the correlation between the activity and different features of the peptides was analyzed in order to determine which characteristics can be altered to improve their activity further. A distributional and positional analysis of the amino acids within the sequences and an univariate ANOVA analysis were used for this purpose. The synthetic peptides showed an increase in activity against both fungi with a higher arginine and tryptophan content, which correlates with findings from previous studies supporting the fact that an increasing balance between the hydrophobicity and the positive charge corresponds with a higher antimicrobial activity. This was further confirmed by the mutual chosen ANOVA descriptor, which was the standard deviation of the Sandberg z1-scale, meaning that high proportions of highly hydrophobic and highly hydrophilic amino acids add to a higher activity in the synthetic peptides.

Furthermore, lysine was found to cause a decrease in activity, especially as a substitution of arginine. The hydrophobic amino acids isoleucine, leucine, tryptophan and valine were detected on position four in strong acting peptides against *C. krusei*, except for valine which causes a decrease in activity. When they appeared on position nine the activity decreased again, except for tryptophan which always increases the activity.

The synthetic peptides against *C.* neoformans showed more or less the same trends as mentioned above for the synthetic peptides against *C. krusei*. There was a more evident decrease in the content of valine on positions two, three, four and five which corresponded to an increase in activity.

The natural peptides obtained a higher activity against the tested fungi when the hydrophobic amino acids tryptophan and isoleucine and the positively charged arginine and lysine were included in their sequences. On the other hand, when glutamine, methionine and the negatively charged glutamic acid and aspartic acid were present in the natural peptides, a lower activity against both fungi was observed. Natural peptides selectively active against *C. krusei* contained more valine, less threonine and the right balance of phenylalanine, proline, serine and leucine. Selective natural peptides against *C. neoformans* contained more cysteine and less glycine and serine. The univariate ANOVA indicated that a peptide sequence of 10 to 17 amino acids and a positive mean charge results in a higher activity for the natural peptides against *C. krusei*.

All the previous assumptions were made for the moderately active and weakly active peptide groups for the synthetic peptides and also for the inactive peptide group for the natural peptides since those groups are comparable in peptide numbers.

The next step is the MIC determination and a hemolysis screen of the highly active peptides. These peptides can then be optimized using the previous analysis results and other techniques and can be screened again until the desired therapeutical potential is obtained.

7. ACKNOWLEDGEMENT

1. World Health Organization, & Who. (2001). WHO Global Strategy for Containment of Antimicrobial Resistance. *World Health*, *WHO/CDS/CS*, 105. Retrieved from http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:WHO+Global+Strategy+for+Containment+of+Antimicrobial+Resistance#0
2. O'Neill, J. (2014). Antimicrobial Resistance : Tackling a crisis for the health and wealth of nations. *Review on Antimicrobial Resistance*, (December).
3. Perea, S., & Patterson, T. F. (2002). Antifungal resistance in pathogenic fungi. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *35*(9), 1073–1080. http://doi.org/10.1086/344058
4. Hilpert, K., Volkmer-Engert, R., Walter, T., & Hancock, R. E. W. (2005). High-throughput generation of small antibacterial peptides with improved activity. *Nature Biotechnology*, *23*(8), 1008–1012. http://doi.org/10.1038/nbt1113
5. Pinheiro Da Silva, F., & MacHado, M. C. C. (2012). Antimicrobial peptides: Clinical relevance and therapeutic implications. *Peptides*. http://doi.org/10.1016/j.peptides.2012.05.014
6. Ashby, M., Petkova, A., & Hilpert, K. (2014). Cationic antimicrobial peptides as potential new therapeutic agents in neonates and children: a review. *Current Opinion in Infectious Diseases*, *27*(3), 258–67. http://doi.org/10.1097/QCO.0000000000000057
7. Nakatsuji, T., & Gallo, R. L. (2012). Antimicrobial Peptides: Old Molecules with New Ideas. *Journal of Investigative Dermatology*. http://doi.org/10.1038/jid.2011.387
8. Phoenix, D. A., Dennison, S. R., & Harris, F. (2013). Antimicrobial Peptides : Their History , Evolution , and Functional Promiscuity. In *Antimicrobial peptides* (pp. 1–37). http://doi.org/10.1002/9783527652853.ch1
9. Brogden, K. A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nature Reviews. Microbiology*, *3*(3), 238–250. http://doi.org/10.1038/nrmicro1098
10. Wang, G., Li, X., & Wang, Z. (2009). ADPD2 - Antimicrobial peptide database. http://doi.org/10.1093/nar/gkn823
11. De Lucca, A. J., & Walsh, T. J. (1999). Antifungal peptides: Novel therapeutic compounds against emerging pathogens. *Antimicrobial Agents and Chemotherapy*, *43*(1), 1–11.
12. Mania, D., Hilpert, K., Ruden, S., Fischer, R., & Takeshita, N. (2010). Screening for antifungal peptides and their modes of action in Aspergillus nidulans. *Applied and Environmental Microbiology*, *76*(21), 7102–7108. http://doi.org/10.1128/AEM.01560-10
13. Loeffler, J., & Stevens, D. A. (2003). Antifungal drug resistance. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *36*(Suppl 1), S31–S41. http://doi.org/10.1086/344658
14. Low, C.-Y., & Rotstein, C. (2011). Emerging fungal infections in immunocompromised patients. *F1000 Medicine Reports*, *3*, 14. http://doi.org/10.3410/M3-14
15. Haney, E. F., & Hancock, R. E. W. (2013). Peptide design for antimicrobial and immunomodulatory applications. *Biopolymers*. http://doi.org/10.1002/bip.22250
16. Guilhelmelli, F., Vilela, N., Albuquerque, P., Derengowski, L. da S., Silva-Pereira, I., & Kyaw, C. M. (2013). Antibiotic development challenges: The various mechanisms of action of antimicrobial peptides and of bacterial resistance. *Frontiers in Microbiology*. http://doi.org/10.3389/fmicb.2013.00353x
17. Van Der Weerden, N. L., Bleackley, M. R., Anderson, M. A. (2013). Properties and mechanisms of action of naturally occurring antifungal peptides. *Cellular and Molecular Life Sciences*.http://doi.org/10.1007/s00018-013-12601
18. Samaranayake, Y. H., & Samaranayake, L. P. (1994). Candida krusei: Biology, epidemiology, pathogenicity and clinical manifestations of an emerging pathogen. *Journal of Medical Microbiology*. http://doi.org/10.1099/00222615-41-5-295
19. Pfaller, M. a., Diekema, D. J., Gibbs, D. L., Newell, V. a., Nagy, E., Dobiasova, S., … Santiago, A. (2008). Candida krusei, a multidrug-resistant opportunistic fungal pathogen: Geographic and temporal trends from the ARTEMIS DISK Antifungal Surveillance Program, 2001 to 2005. *Journal of Clinical Microbiology*, *46*(2), 515–521. http://doi.org/10.1128/JCM.01915-07
20. Gubbins, P. O., Anaissie, E. J., McGinnis, M. R., & Pfaller, M. A. (2009). *Candida.* In *Clinical Mycology 2nd ed* (pp. 195–239).
21. Gubbins, P. O., Anaissie, E. J., McGinnis, M. R., & Pfaller, M. A. (2009). *Cryptococcus.* In *Clinical Mycology 2nd ed* (pp. 240–259).
22. Bose, I., Reese, A. J., Ory, J. J., Doering, T. L., & Janbon, G. (2003). A Yeast under Cover : the Capsule of Cryptococcus neoformans, *2*(4), 655–663. http://doi.org/10.1128/EC.2.4.655
23. Archibald, L. K., Tuohy, M. J., Wilson, D. A., Nwanyanwu, O., Kazembe, P. N., Tansuphasawadikul, S., … Procop, G. W. (2004). Antifungal Susceptibilities of Cryptococcus neoformans. *Emerging Infectious Diseases*, *10*(1), 143–145. http://doi.org/10.3201/eid1001.020779
24. Loftus, B. J., Fung, E., Roncaglia, P., Rowley, D., Amedeo, P., Bruno, D., … Hyman, R. W. (2005). The genome of the basidiomycetous yeast and human pathogen Cryptococcus neoformans. *Science (New York, N.Y.)*, *307*(5713), 1321–1324. http://doi.org/10.1126/science.1103773
25. Sorrell, T. C., & Ellis, D. H. (1997). Ecology of Cryptococcus neoformans. *Revista Iberoamericana de Micologia : Organo de La Asociacion Espanola de Especialistas En Micologia*, *14*(2), 42–43. http://doi.org/19971442 [pii]
26. Capenberghs, S. (2011). *Vaste-fase Synthese van Sequentie Gecontroleerde Polymeren op Basis van Thiol " Click " Chemie.*
27. Hindriks, R. (1973). *Vaste-fase peptidesynthesen: Synthese van oxytocine, vasopressine en menselijk calcitonine met behulp van een machine.*
28. Duro-Castano, A., Conejos-Sánchez, I., & Vicent, M. (2014). Peptide-Based Polymer Therapeutics. *Polymers*, *6*(2), 515–551. http://doi.org/10.3390/polym6020515
29. Carpino, L. A., & Han, G. Y. (1972). The 9-Fluorenylmethoxycarbonyl Amino-Protecting Group. *J Org Chem*, *37*(22), 3404–3409. http://doi.org/10.1021/jo00795a005
30. Winkler, D. F. H., & Campbell, W. D. (2008). The spot technique the spot technique: Synthesis and screening of peptide macroarrays on cellulose membranes.
31. Frank, R. (2002). The SPOT-synthesis technique: Synthetic peptide arrays on membrane supports - Principles and applications. *Journal of Immunological Methods*, *267*(1), 13–26. http://doi.org/10.1016/S0022-1759(02)00137-0
32. Hilpert, K., Winkler, D. F. H., & Hancock, R. E. W. (2007). Peptide arrays on cellulose support: SPOT synthesis, a time and cost efficient method for synthesis of large numbers of peptides in a parallel and addressable fashion. *Nature Protocols*, *2*(6), 1333–1349. http://doi.org/10.1038/nprot.2007.160
33. Arendrup, M., Hope, W., Lass-Flörl, C., Cuenca-Estrella, M., Arikan-Akdagli, S., Barchiesi, F., Verweij, P., the Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST). (2012). Method for the determination of broth dilution minimum Inhibitory concentrations of antifungal agents for yeasts. *Clinical Microbiology and Infection*, *18*(7), 1–21..
34. Wiegand, I., Hilpert, K., & Hancock, R. E. W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*, *3*(2), 163–175. http://doi.org/10.1038/nprot.2007.521
35. O'Brien, J., Wilson, I., Orton, T., & Pognan, F. (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *European Journal of Biochemistry*, *267*(17), 5421–5426. http://doi.org/10.1046/j.1432-1327.2000.01606.x
36. Borra, R. C., Lotufo, M. A., Gagioti, S. M., Barros, F. D. M., & Andrade, P. M. (2009). A simple method to measure cell viability in proliferation and cytotoxicity assays. *Brazilian Oral Research*, *23*(3), 255–262. http://doi.org/10.1590/S1806-83242009000300006
37. Promega Corporation. (2013). CellTiter-Blue® Cell Viability Assay Protocol. *Promega.com*, 1–15. Retrieved from https://www.promega.com/~/media/Files/Resources/Protocols/Technical Bulletins/101/CellTiter-Blue Cell Viability Assay Protocol.pdf
38. Fuller, B. J. (2004). Cryoprotectants: The essential antifreezes to protect life in the frozen state. *Cryo-Letters*.
39. Mikut, R. (2010). Computer-based analysis, visualization, and interpretation of antimicrobial peptide activities. *Methods in Molecular Biology (Clifton, N.J.)*, *618*, 287–299. http://doi.org/10.1007/978-1-60761-594-1_18

40. Mikut, R., Reischl, M., Ulrich, A., & Hilpert, K. (2008). Data-Based Activity Analysis and Interpretation of Small Antibacterial Peptides. *Proceedings of 18th Workshop on Computational Intellengence*, 189–203. Retrieved from http://wwwserv1.iai.fzk.de/www-extern-kit/fileadmin/Image_Archive/Hochdurchsatz/biosignal/publikationen/mikut08dort_peptide.pdf
41. Data, P., & Data, P. (2008). EUCAST technical note on fluconazole. *Clinical Microbiology and Infection : The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *14*(2), 193–195. http://doi.org/10.1111/j.1469-0691.2007.01899.x
42. International Union of Pure And Applied Chemistry, ., & International Union of Biochemistry, . (1971). International Union of Biochemistry a One-Letter Notation for Amino Acid Sequences (Definitive Rules) a One-Letter Notation for Amino Acid. *IUPAC-IUB Commission on Biochemical Nomenclature*, 639–645.
43. Mikut, R., Ruden, S., Reischl, M., Breitling, F., Volkmer, R., Hilpert, K. (2015). Improving short antimicrobial peptides despite elusive rules of activity. Manuscript submitted for publication.
44. Kang, S. J., Won, H. S., Choi, W. S., & Lee, B. J. (2009). De novo generation of antimicrobial LK peptides with a single tryptophan at the critical amphipathic interface. In *Journal of Peptide Science* (Vol. 15, pp. 583–588). http://doi.org/10.1002/psc.1149
45. Wang, H., Yu, Z., Hu, Y., Li, F., Liu, L., Zheng, H., … Liu, J. (2012). Novel antimicrobial peptides isolated from the skin secretions of Hainan odorous frog, Odorrana hainanensis. *Peptides*, *35*(2), 285–290. http://doi.org/10.1016/j.peptides.2012.03.007
46. Muta, T., Fujimoto, T., Nakajima, H., & Iwanaga, S. (1990). Tachyplesins isolated from hemocytes of Southeast Asian horseshoe crabs (Carcinoscorpius rotundicauda and Tachypleus gigas): identification of a new tachyplesin, tachyplesin III, and a processing intermediate of its precursor. *Journal of Biochemistry*, *108*(2), 261–266.
47. Nakamura, T., Furunaka, H., Miyata, T., Tokunaga, F., Muta, T., Iwanaga, S., … Shimonishi, Y. (1988). Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (Tachypleus tridentatus). Isolation and chemical structure. *Journal of Biological Chemistry*, *263*(32), 16709–16713.
48. Romeo, D., Skerlavaj, B., Bolognesi, M., & Gennaro, R. (1988). Structure and bactericidal activity of an antibiotic dodecapeptide purified from bovine neutrophils. *Journal of Biological Chemistry*, *263*(20), 9573–9575.
49. Rozek, T., Wegener, K. L., Bowie, J. H., Olver, I. N., Carver, J. A., Wallace, J. C., & Tyler, M. J. (2000). The antibiotic and anticancer active aurein peptides from the Australian Bell Frogs Litoria aurea and Litoria raniformis: The solution structure of aurein 1.2. *European Journal of Biochemistry*, *267*(17), 5330–5341. http://doi.org/10.1046/j.1432-1327.2000.01536.x
50. Yin, L. M., Edwards, M. A., Li, J., Yip, C. M., & Deber, C. M. (2012). Roles of hydrophobicity and charge distribution of cationic antimicrobial peptides in peptide-membrane interactions. *Journal of Biological Chemistry*, *287*(10), 7738–7745. http://doi.org/10.1074/jbc.M111.303602
51. Chan, D. I., Prenner, E. J., & Vogel, H. J. (2006). Tryptophan- and arginine-rich antimicrobial peptides: Structures and mechanisms of action. *Biochimica et Biophysica Acta - Biomembranes.* http://doi.org/10.1016/j.bbamem.2006.04.006
52. Mahalakshmi, R., Franzin, C. M., Choi, J., & Marassi, F. M. (2007). NMR structural studies of the bacterial outer membrane protein OmpX in oriented lipid bilayer membranes. *Biochimica et Biophysica Acta*, *1768*(12), 3216–3224. http://doi.org/10.1016/j.bbamem.2007.08.008
53. Lee, J., & Lee, D. G. (2010). Influence of the hydrophobic amino acids in the N- and C-terminal regions of pleurocidin on antifungal activity. *Journal of Microbiology and Biotechnology*, *20*(8), 1192–1195. http://doi.org/10.4014/jmb.1004.04041
54. Johnsen, L., Fimland, G., Eijsink, V., & Nissen-Meyer, J. (2000). Engineering increased stability in the antimicrobial peptide pediocin PA-1. *Applied and Environmental Microbiology*, *66*(11), 4798–4802. http://doi.org/10.1128/AEM.66.11.4798-4802.2000
55. Pellegrini, A., Thomas, U., Bramaz, N., Klauser, S., Hunziker, P., & von Fellenberg, R. (1997). Identification and isolation of a bactericidal domain in chicken egg white lysozyme. *J Appl Microbiol*, *82*(3), 372–378. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12455902
56. Broekaert, W. F., Mariën, W., Terras, F. R., De Bolle, M. F., Proost, P., Van Damme, J., … Vanderleyden, J. (1992). Antimicrobial peptides from Amaranthus caudatus seeds with sequence homology to the cysteine/glycine-rich domain of chitin-binding proteins. *Biochemistry*, *31*(17), 4308–4314. http://doi.org/doi: 10.1021/bi00132a023
57. Sandberg, M., Eriksson, L., Jonsson, J., Sjöström, M., & Wold, S. (1998). New chemical descriptors relevant for the design of biologically active peptides. A multivariate characterization of 87 amino acids. *Journal of Medicinal Chemistry*, *41*(14), 2481–2491. http://doi.org/10.1021/jm9700575
58. Guilhelmelli, F., Vilela, N., Albuquerque, P., Derengowski, L. da S., Silva-Pereira, I., & Kyaw, C. M. (2013). Antibiotic development challenges: The various mechanisms of action of antimicrobial peptides and of bacterial resistance. *Frontiers in Microbiology*. http://doi.org/10.3389/fmicb.2013.00353
59. Larson, B., Held, P., & Banks, P. (n.d.). Improving Fluorescence-Based Assay Performance Using Automated Digital Microscopy Phenotypic Assay Analysis in Cells Live Cell Assay Analysis using a 3D Spheroid Model, 5404.
60. PerkinElmer. (n.d.). Microplates for fluorescence assays. Retrieved May 7, 2015, from http://www.perkinelmer.com/resources/technicalresources/applicationsupportknowledgebase/microplates/fluorescence_plates.xhtml
61. Cherkasov, A., Hilpert, K., Jenssen, H., Fjell, C. D., Waldbrook, M., Mullaly, S. C., … Hancock, R. E. W. (2009). Use of artificial intelligence in the design of small peptide antibiotics effective against a broad spectrum of highly antibiotic-resistant superbugs. *ACS Chemical Biology*, *4*(1), 65–74. http://doi.org/10.1021/cb800240j
62. Cuenca-Estrella, M., Gomez-Lopez, a., Cuesta, I., Zaragoza, O., Mellado, E., & Rodriguez-Tudela, J. L. (2011). Frequency of voriconazole resistance in vitro among Spanish clinical isolates of Candida spp. according to breakpoints established by the antifungal subcommittee of the European Committee on Antimicrobial Susceptibility Testing. *Antimicrobial Agents and Chemotherapy*, *55*(4), 1794–1797. http://doi.org/10.1128/AAC.01757-10
63. Winkler, D. F. H., & Hilpert, K. (2010). Synthesis of antimicrobial peptides using the SPOT technique. *Methods in Molecular Biology (Clifton, N.J.)*, *618*, 111–124. http://doi.org/10.1007/978-1-60761-594-1_8
64. Souza, L. K. H., Fernandes, O. D. F. L., Kobayashi, C. C. B. A., Passos, X. S., Costa, C. R., Lemos, J. A., … Silva, M. D. R. R. (2005). Antifungal susceptibilities of clinical and environmental isolates of Cryptococcus neoformans in Goiânia City, Goiás, Brazil. *Revista Do Instituto de Medicina Tropical de Sao Paulo*, *47*(5), 253–256. http://doi.org/10.1590/S0036-46652005000500003
65. Fleming, A. (2007). Zebrafish as an alternative model organism for disease modelling and drug discovery : implications for the 3Rs. NC3Rs, 10(Figure 1), 1–7. http://doi.org/www.nc3rs.org.uk